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THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE BIOLOGICAL BULLETIN

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THE FATE OF THE ECTODERM AND ENDODERM OF HYDRA WHEN CULTURED INDEPENDENTLY

E. J. PAPENFUSS AND N. A. H. BOKENHAM¹

(From the Zoölogical Laboratory of the University of Cape Town)

It was recently found by Gilchrist (1937) that pieces of ectoderm of scyphozoan polyps, presumably the scyphistomas of *Aurellia*, are able to regenerate small complete polyps; while endodermal pieces are unable to regenerate although they may remain alive for several days.

It is of considerable interest that a complete polyp can be regenerated from ectoderm alone as this phenomenon necessitates the formation of the endodermal layer from a tissue composed of cells of a completely different structure. In view of this fact it becomes of importance to know whether the newly regenerated tissue layer is formed by a transformation of certain of the differentiated cells; or whether undifferentiated embryonic cells are activated to form anew the characteristic cell types of the lacking tissue, as Wilson and Penney (1930) found to be true for many cell types in regeneration from dissociated sponge tissue.

Gilchrist did not study the origin of the endodermal layer in his regenerates but suggests that it may have been formed by the interstitial cells of the ectoderm. The present study was undertaken with the object of casting light upon this question. *Hydra* is a favorable organism for studying this problem as the endoderm can readily be separated from the ectoderm in the living condition (Papenfuss, 1934, pp. 227-228). Accordingly the two cellular layers were isolated and cultured independently. Both green and brown *Hydra* (species undetermined) were employed in each series of experiments.

Attempts to Obtain Regeneration from the Ectodermal Layer

In order to obtain a *Hydra* consisting only of ectoderm, it was first necessary to cut off the head of the specimen since it was not possible to remove the endoderm from the tentacles. The specimen was next everted and the endoderm removed. The *Hydra* now consisting only

¹ The experimental phase of this study was carried out by E. J. Papenfuss while the histological part was done by N. A. H. Bokenham.

of the ectodermal layer was then carefully examined under a higher magnification to make certain that fragments of endoderm had not remained behind. Hydras with buds were not used as the endoderm could not be removed from these parts. In order to be certain that the entire endodermal layer is removed by this method, paraffin sections were made of individuals which had been so treated. Such sections show the specimens to be without endoderm. For the histological phase of the work, the specimens were fixed in formol-acetic-acid, stained in safranin or Heidenhain's haematoxylin, and fast green. The latter was found to be a good stain for the mesogloal layer.

After the endoderm had been removed from the *Hydra* which was to be cultured, the specimen was placed in a small Stender dish containing pond water. Filtered pond water was used throughout the experiments, and all the dishes and instruments were kept chemically clean. In order to ascertain if the conditions were favorable for the cultured tissue, a normal *Hydra* was placed in each dish.

The data for this part of the paper were taken from 30 consecutive experiments. At the beginning of the study a few trial experiments were made which were not taken into consideration in the compilation of the results.

In none of the experiments in which the ectoderm alone was cultured did a *Hydra* develop. The tissue always disintegrated, while the controls lived and appeared entirely normal. In 14 of the experiments the ectodermal tissue had completely disintegrated within 24 hours, in 8 of the experiments the tissue did not disintegrate until the second day, in 7 of the experiments a small piece of tissue persisted until the third day, and in one case a minute fragment of what appeared to be ectodermal tissue persisted until the seventh day.

It may be noted that Gilchrist used only pieces of ectodermal tissue in his experiments while in the present work the entire ectodermal layer with the exception of the ectoderm of the head was used, as it seemed likely that regeneration would occur more readily from a large piece of tissue than from a small one.

Since it was necessary in these experiments to remove the head of the *Hydra* and to evert the body in order to make the endoderm accessible, a series of experiments were performed to determine whether a *Hydra* so treated can live and regenerate. The results from 35 experiments may be summarized as follows: In 30 of the experiments the *Hydra* lived and became normal; that is, the ectoderm again formed the external layer and tentacles developed. In the remaining five experiments, the *Hydra* died. In practically all the experiments in which the *Hydra* lived, the ectoderm was again on the exterior within 24 hours.

The results of these experiments thus show that the majority of hydras will live under such conditions although an occasional one may die. It may be noted that in the experiments in which the *Hydra* did not live, the specimen was badly damaged while it was being everted. In the experiments in which the isolated ectodermal layer was cultured, such specimens were discarded as it was then difficult to remove the endoderm.

From the first series of these experiments it may be concluded that the ectodermal layer of *Hydra* alone is not able to regenerate an individual, while the second series of experiments shows that the failure of the ectodermal layer to live and regenerate cannot be attributed to the treatment the *Hydra* had received prior to the removal of the endodermal layer. In order to determine whether the isolated endodermal tissue is capable of regeneration, the experiments set forth in the following section were performed.

Attempts to Obtain Regeneration from Endodermal Tissue

It was not possible to obtain a complete endodermal layer by removing the ectoderm as the latter is rigid and difficult to cut. It was accordingly necessary to evert the *Hydra* and to remove the endoderm by scraping it off with a fine glass needle. This tissue, however, dissociates very readily and comes off in small fragments. Since Peebles (1897) has shown that the smallest portion of a *Hydra* that is capable of regeneration is a sphere whose diameter is $\frac{1}{6}$ mm., it was considered necessary to fuse the fragments together so that a piece of tissue larger than this minimum size could be obtained for culturing. In the green species the fusion masses measured from 0.8 mm.—0.9 mm. in diameter and in the brown form they measured from 0.9 mm.—1.2 mm. in diameter. In a few experiments with both the green and the brown *Hydra*, the endodermal fragments from two individuals of the same species were allowed to fuse in order to obtain a larger mass of tissue.

As the endodermal tissue of the green *Hydra* dissociates more readily than that of the brown, it was necessary to employ somewhat different methods for the two forms. When working with the green *Hydra*, the fragments were transferred by means of a fine pipette to a small hole in an agar bed. They were then placed as close together as possible so as to be in a position favorable for fusion. Only a small amount of water was allowed on the agar to prevent the fragments from floating apart. The slide was examined frequently and water added when necessary to prevent the tissue from becoming dry. If the hydras were fresh from the pond, complete fusion occurred in this form in approximately 15

minutes. Hydras which had been in the laboratory for some time were unsuitable for this type of experiment unless they were from a well-established culture.

When brown hydras were used it was found that larger fragments of endoderm could be obtained as the endoderm in this form dissociates less readily than in the green *Hydra*. It was then more practicable to place each individual fragment in contact with the other fragments as soon as it was removed from the *Hydra*. This was done with a fine glass needle. As much water as possible was withdrawn to prevent the fragments from floating apart. As the process of fusion takes longer in the brown *Hydra*, the slide was kept in a damp chamber, examined frequently, and water added when necessary to prevent the tissue from becoming dry.

After the tissue had fused securely, the fusion mass was taken up in a pipette with a comparatively large opening and transferred to a small dish containing filtered spring water. A normal *Hydra*, to be used as a control, was placed in the dish with the endodermal fusion mass.

This type of experiment is difficult to perform since the endoderm easily dissociates. The writers, however, carried through to completion 12 experiments. In no case was a new *Hydra* regenerated from the endodermal tissue, although the controls lived and appeared entirely normal. In the majority of experiments the fusion mass had completely disintegrated within 24 hours while in a few cases minute fragments of the tissue could still be recognized after two or three days.

The results of these experiments show that the endoderm of *Hydra* alone is not capable of regenerating an individual. It is of interest to note that similar fusion masses composed of both ectoderm and endoderm will regenerate complete new hydras (Issajew, 1926; Papenfuss, 1932, 1934; Weimer, 1934).

Discussion and Conclusions

The object of the present study was to ascertain if a *Hydra* can be regenerated from either ectoderm or endoderm alone. The results of the experiments were entirely negative.

Since *Hydra* has great regenerative abilities, being able to form a new individual from a small fragment of tissue (composed of both ectoderm and endoderm) only $\frac{1}{6}$ mm. in diameter (Peebles, 1897), it is of considerable interest that a regenerate is not formed when practically an entire cellular layer is cultured. In attempting to explain these results, the writers suggest that a regenerate did not develop because

the differentiated cells of one tissue layer are unable to transform into the cell types characteristic of the lacking tissue layer, and the undifferentiated cells (the embryonic interstitial cells) do not become activated to regenerate this tissue.

That differentiated cells do not transform into different cell types has been shown to be true previously by Wilson and Penney (1930), who found that in reunion of sponge tissue from dissociated cells, the specialized cells (the collar cells) which are carried over into the reunion mass do not change their characteristics but become the collar cells of the new individual. Likewise in reunion in *Hydra* the specialized foot cells retain their identity and function as the foot cells of the new individual (Papenfuss, 1934).

As the unspecialized interstitial cells of *Hydra* which are present in both the ectoderm and the endoderm are known to possess certain formative powers (Kleinenberg, 1872; Schneider, 1890; McConnell, 1932; and others), it seemed possible that these cells might regenerate anew the lacking tissue layer. However, judging from our results, it may be concluded that the formative powers of the interstitial cells are limited. According to Kanajew (1930), the interstitial cells play a subordinate rôle in regeneration in *Hydra*, the regenerated part, at least as regards the endoderm, being formed at the expense of the nearby differentiated tissue. In the sponge, however, the unspecialized mesenchyme cells possess great formative powers, being able to reestablish very many of the characteristic cell types of a regenerate (Wilson and Penney, 1930).

It should be noted that Ischikawa (1890) previously attempted to obtain *Hydra* regenerates from ectoderm alone. This author obtained ectodermal tissue by destroying the endoderm with acetic acid vapor. No regenerates were formed, however, and Ischikawa concluded that the interstitial cells were unable to give rise to the lacking tissue layer. Schulze (1918) suggests that the failure of the ectodermal tissue to regenerate was owing to the lack of nourishment normally supplied by the endoderm. Schulze's conclusion, however, loses support by the fact that isolated endodermal tissue also fails to regenerate, as was found in the present study.

The results of our experiments are at variance with those of Gilchrist (1937), who found that pieces of ectoderm of a scyphistoma are able to regenerate complete new individuals. As the disposition of the endoderm of a scyphozoan polyp is somewhat complicated by the invasion of the ectodermal cells in the four taeniolae, it is possible that Gilchrist did not succeed in removing all the endoderm from the fragments.



Acknowledgment

The writers wish to thank Professor W. A. Jolly for the loan of a dissecting microscope and for granting them the facilities of the Department of Physiology where part of the work was done. They further wish to thank Dr. M. A. Pocock for aid in the collecting of material.

BIBLIOGRAPHY ²

- GILCHRIST, FRANCIS G., 1937. Budding and locomotion in the scyphistomas of Aurelia. *Biol. Bull.*, **72**: 99.
- *ISCHIKAWA, C., 1890. Trembley's Umkehrungsversuche an Hydra nach neuen Versuchen erklärt. *Zeitschr. f. wissenschaft. Zool.*, **49**: 433.
- ISSAJEW, W., 1926. Studien an organischen Regulationen. (Experimentelle Untersuchungen an Hydren.) *Arch. f. Entw.-mech. Organism.*, **108**: 1.
- *KANAJEW, J., 1930. Zur Frage der Bedeutung der interstitiellen Zellen bei Hydra. *Arch. f. Entw.-mech. Organism.*, **122**: 736.
- KLEINENBERG, N., 1872. Eine anatomisch entwicklungsschichtliche Untersuchung. Leipzig.
- MCCONNELL, C. H., 1932. The development of the ectodermal nerve net in the buds of Hydra. *Quart. Jour. Micr. Sci.*, **75**: 495.
- PAPENFUSS, E. J., 1932. Experimental studies on fusion in Hydra (Abstract). *Anat. Rec.*, **54**: 54 (Suppl.).
- PAPENFUSS, E. J., 1934. Reunion of pieces in Hydra, with special reference to the rôle of the three layers, and to the fate of differentiated parts. *Biol. Bull.*, **67**: 223.
- PEEBLES, FLORENCE, 1897. Experimental studies on Hydra. *Arch. f. Entw.-mech. der Organism.*, **5**: 794.
- SCHNEIDER, K. C., 1890. Histologie von Hydra fusca mit besonderer Berücksichtigung des Nervensystems der Hydropolyten. *Arch. mikr. Anat.*, **35**: 321.
- SCHULZE, P., 1918. Die Bedeutung der interstitiellen Zellen für die Lebensvorgänge bei Hydra. *Sitzgsber. Ges. Naturforsch. Freunde Berlin*, Nr. 7: 252.
- WEIMER, B. R., 1934. The physiological gradients of Hydra. III. Reconstitution of masses of dissociated pieces. *Physiol. Zoöl.*, **7**: 212.
- WILSON, H. V., AND J. T. PENNEY, 1930. The regeneration of sponges (Microciona) from dissociated cells. *Jour. Exper. Zoöl.*, **56**: 73.

² The authors did not have access to the papers marked with an asterisk but have seen the abstract of Kanajew's work in *Biological Abstracts*, Vol. 5 (II), 1931, p. 2952; and have read Schulze's (1918) account of the work of Ischikawa.

THE REPRODUCTIVE SYSTEM AND SPERMATOGENESIS OF LIMACINA (SPIRATELLA) RETROVERSA (FLEM.)¹

SIDNEY C. T. HSIAO

(From the Biological Laboratories, Harvard University and the Woods Hole Oceanographic Institution,² Woods Hole, Massachusetts)

INTRODUCTION

Limacina retroversa was described as early as 1823 by Fleming under the name of "*Heterofusus retroversus*," but very little is known about its internal structure, its method of reproduction and its life history. Writing in 1924—one hundred years later—Bigelow mentioned that "Nothing is known of the reproduction of *Limacina retroversa* in the Gulf of Maine. . . ." The embryonic development and the reproductive system of several species of Pteropoda have been described but no exact information on the species *L. retroversa* is available. Van Beneden described the structure of the reproductive system of *L. arctica* in 1839 but, as pointed out by Meisenheimer (1905), he made the mistake of calling the ovotestis the ovary, the vesicula seminalis the testis and the accessory glands the "gland prostatique." Studies of the embryonic development of pteropods have been made by Gegenbaur (1853, 1855), Krohn (1856), Müller (1857), Fol (1874, 1875) and Knipowitsch (1891). Meisenheimer (1905) described the comparative morphology of Pteropoda and compared the genus *Limacina* with other Euthecosomata. He considered *Limacina* as a general group and made no mention of any species. Meisenheimer also appraised the earlier work and observations of Cuvier, d'Orbigny, Van Beneden, Gegenbaur, Souleyet, Pelseneer, Knowler, etc. Bonnevie (1916) made further observations on the reproductive organs of *Cuvierina* and recently Zarnik (1911) determined the chromosome number of several species of pteropods. Up to the present no one has studied the entire life history of any species of Pteropoda and it has been unknown whether any pteropods, like some other mollusks, change their sex. The reproductive system of *Limacina retroversa*, in particular, has not been studied. It

¹ The name "*Limacina retroversa*" has been in use until 1930, when Sherborn pointed out that Cuvier (1817) used the vernacular name "Limacine" and he restored de Blainville's "*Spiratella*" in place of Lamarck's (1819) "*Limacina*." Thiele (1931) also used "*Spiratella*." However, as "*Limacina*" has the advantage of being more commonly used and better known it is retained in this paper.

² Contribution No. 197 from the Woods Hole Oceanographic Institution.

is the purpose of this paper to describe the reproductive system and spermatogenesis of *Limacina retroversa* as a basis for a report on the reproductive history of this species to appear in a subsequent paper.

MATERIAL AND METHODS

The material was collected by the research vessel "Atlantis" of the Woods Hole Oceanographic Institution during a series of cruises in the Gulf of Maine between June, 1933 and September, 1934. The specimens were caught by vertical hauls made with a 1.5-meter Heligoland net drawn from a level near the bottom up to the surface and were fixed on board the ship in 2 per cent formalin in sea water.

Each specimen used for making permanent mounts was decalcified in 1 per cent acetic acid for a few minutes. In some cases the acid in the commercial formalin used in fixation had decalcified the specimens. The decalcified animal was then stained in toto for about one minute in a 0.1 per cent aqueous solution of Ehrlich's methylene blue chloride to aid in orienting it in the paraffin block for sectioning. The stained specimen was dehydrated in two grades of 1-4 dioxene in a calcium tower and directly imbedded in 53°-55° C. paraffin. Each individual was cut in serial longitudinal sections five micra in thickness which were mounted in order on slides. The vital stain was removed when the sections were hydrated after the removal of paraffin with xylene. Standard methods of staining with Heidenhain's iron haematoxylin and orange G were used in the final staining process. Stained sections were cleared in xylene and mounted in Canadian balsam.

In order to reconstruct the different parts of the reproductive system sections from suitable individuals were drawn with a camera lucida. The tracings of every fifth or tenth section were made with a wax pencil on pieces of glass. Reconstruction of the reproductive system was made by superimposing the glass plates in order so as to determine the size and shape of each organ. These tracings were compared with Meisenheimer's plates and descriptions (1905) of similar sections of the reproductive apparatus of Euthecosomata. Meisenheimer's terminology of the different parts of the regenerative system was followed.

In the study of the spermatogenesis of *Limacina retroversa* the method for carmine smears was used in addition to the examination of permanent mounts stained in Heidenhain's haematoxylin. The specimens used for making carmine smears were collected in the summer of 1937 near Woods Hole. As soon as the animals were taken from water they were fixed for twenty minutes in a mixture of one part of acetic acid and three parts of absolute alcohol. Each *Limacina* was sepa-

rated from other planktonic material and stored in 70 per cent alcohol. It was necessary to change the preservative several times in order to get rid of the precipitate brought down by alcohol from sea water. The gonads from several individuals were dissected out under a binocular microscope and torn up into very small pieces. They were then stained with cytological aceto-carmin for a few minutes, covered with a coverglass and heated over a small flame without boiling. The slide with the material was then pressed between blotting papers to separate the cells and to remove the excessive stain. Such preparations showed the cellular structures very clearly and were used for spermatogenesis studies.

Living animals have been kept in the laboratory for a week to ten days for observation of spawning and egg-development.

OBSERVATIONS

The Reproductive System

The reproductive system of *Limacina retroversa* consists of a bisexual gonad, a gonoduct, accessory glands and a penis.

The Gonads.—The bisexual gonad or ovotestis constitutes the primary sexual organ. In a mature individual it is a spiral structure situated posterior to the liver and occupies the posterior or last three or four whorls of the shell with whose cavity the smooth, non-lobated coils of the ovotestis conform (Fig. 1). In specimens preserved in formalin the gonad appears, after decalcification, granular and pinkish in contrast to the yellowish-green liver with which it is continuous. In living specimens the gonads can also be distinguished from the greenish liver through the transparent shell. In sectional view, the cortical and apical regions of the mature gonads are generally occupied by the female reproductive cells and the medullary portion by the spermatatic tissues. The spermatatic tissues are apparently arranged in tubules in the medulla of the ovotestis, but in well-developed males they may extend to the cortex with masses of mature spermatozoa obliterating the tubular arrangement of the reproductive cells. In large females, on the other hand, the mature ova may be found in the medullary region just before they pass into the hermaphroditic duct. The proportion of masculine and feminine reproductive cells varies with the season as well as with the size of the animal.

Three different types of gonads can be distinguished. (A) Sexually undifferentiated gonads all of which occur in small-sized specimens; (B) "pure male" gonads which belong to somewhat larger animals but never to animals greater than 1.1 mm. in diameter across the largest



whorl of the shell; and (C) hermaphroditic gonads, or ovotestes, characteristic of all larger individuals.

A. Sexually undifferentiated gonads.—In very small specimens the gonad is sexually undifferentiated in that no definite male or female reproductive cells can be recognized. This type of gonad corresponds with the "indefinite gonads" of Coe (1931) and Orton (1926). The whole gonad is small, nearly spherical and surrounded by the liver. The pregerminative cells, all of nearly equal size, form a solid mass which is enclosed within a thin fibrous Ancel's layer consisting of elongated cells

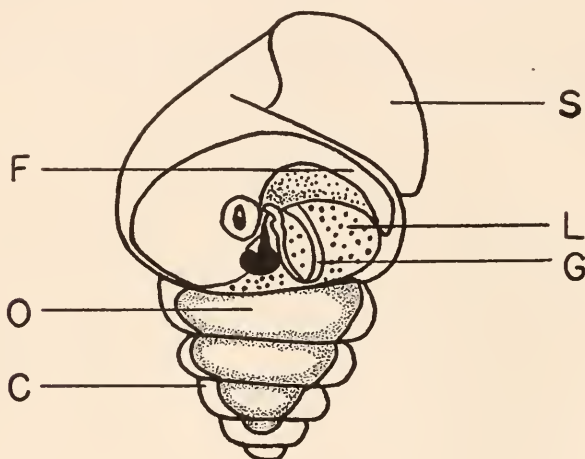


FIG. 1. Camera lucida drawing of *Limacina retroversa* ($\times 30$), showing the gonad and the rest of the body.

- C. Cavity between shell and ovotestis.
- F. "Flapper" or "foot."
- G. Gut.
- L. Liver.
- O. Ovotestis.
- S. Shell opening.

with prominent nuclei. The cellular structure of the pregerminative cells is not easy to make out, for in specimens fixed with commercial formalin and stained with haematoxylin the nuclei are diffused and the structure not clear. In this, as in the other types of gonads, the fixative used tends to make the cells indistinct from each other so that they have the appearance of a syncytium. A sectional view of a young indefinite gonad is shown in Fig. 2, *A*.

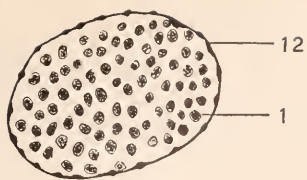
B. "Pure male" gonads.—All *Limacina retroversa* larger than 0.85 mm. in diameter show sexual differentiation, for either male or female

germ cells are discernible. But nearly all, if not all, of the very small sexually differentiated individuals are protandric. Half of the protandric animals do not show any ovogenesis at all. Individuals possessing this type of gonad correspond to Coe's (1933) "true males" or Orton's (1909) "pure males." In this description they will be called "pure males." The "pure male" type of gonad is confined to small-sized *Limacina retroversa*. No individuals larger than 1.1 mm. in diameter show complete maleness in their gonadal organization. In Fig. 2, *B* a gonad without any feminine reproductive cells distinguishable from the male cells is shown. In this diagram a few clusters of spermatozoa and a few groups of spermatids are seen while many of the other cells are undergoing active spermatogenesis.

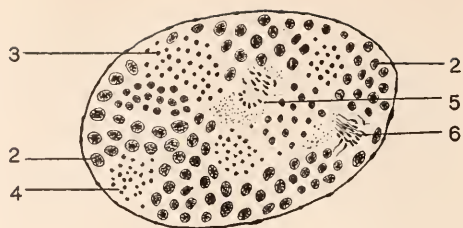
C. Hermaphroditic gonads.—Besides "pure males" sexually differentiated *Limacina* have gonads with various proportions of masculine and feminine types of tissue and show different signs of sexual activity. For convenience of description and comparison, these larger specimens, possessing ovotestes, can be divided into classes in terms of the percentage of masculine or feminine tissue in the gonad. Individuals with more than 50 per cent of the tissue of their gonads consisting of masculine reproductive cells, showing spermatogenesis, are designated as hermaphroditic males and those with less than 50 per cent of the contents of their gonads made of male reproductive cells are called hermaphroditic females. On this percentage basis the gonads can be arranged from extreme masculine to pronounced feminine types.

Gonads of individuals which are classified as hermaphroditic males are shown in Fig. 2, *C* and *D*. Figure 2, *C* shows the gonad of a mature hermaphroditic male with more than 75 per cent of its reproductive tissues consisting of masculine cells. This gonad occurred in a *Limacina* 1.2 mm. in diameter. In contrast to the "pure male" type of gonad, a peripheral layer of ovarian tissue is present, but it amounts to less than 25 per cent of the total reproductive cells in the organ. In the medullary portion clusters of mature spermatozoa are present while in the cortical region a small number of egg cells are developing. Individuals possessing this type of gonad may be of any size but most of them are comparatively small. They are smaller than the pronounced feminine type but larger than the "pure males."

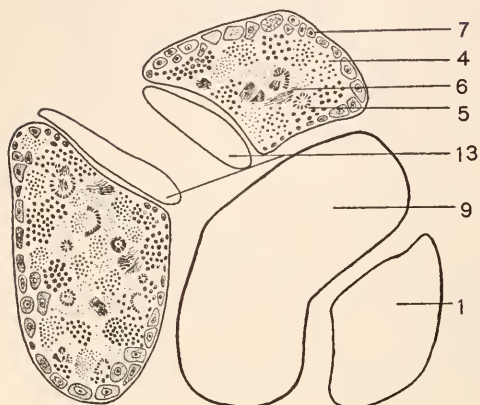
Figure 2, *D* shows the ovotestis of an individual belonging to the class having one-half to three-quarters of the gonad occupied by male tissue. In this ovotestis mature cells of both sexes are seen. At a point near the beginning of gonoduct, next to the retractor muscles, in the largest whorl of the spiral organ, mature ova and spermatozoa are seen ready to leave the sex gland. This simultaneous presence of ma-



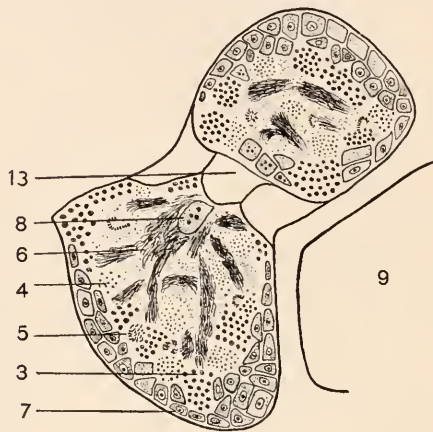
A



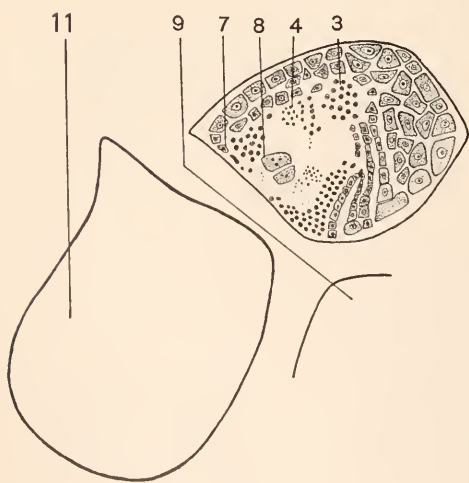
B



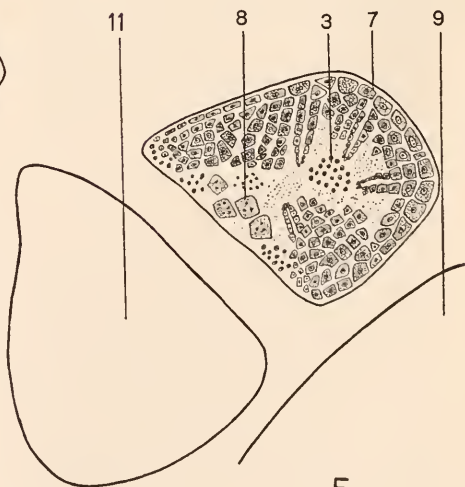
C



D



E



F

FIGURE 2

ture ova and spermatozoa near the gonoduct, or the presence of mature cells of both sexes along it, indicates that the animal is functioning both as male and female. Individuals of this type are called functional hermaphrodites, a name commonly used to designate this kind of reproductive individual among mollusks.

Animals with less than half of the gonadal tissues consisting of male reproductive cells, which are classified as hermaphroditic females, can also be divided into two groups in a similar manner: (a) hermaphroditic females with gonads containing 50–25 per cent of male tissue, and (b) hermaphroditic females with less than 25 per cent of male tissue in the gonad. These two types are represented in Fig. 2, *E* and *F*. As a general rule, gonads which are predominantly feminine are in a mature stage, containing mature ova and very often both mature ova and spermatozoa. The *Limacina* possessing these gonads are generally larger than the masculine types.

Part of a gonad with 25–50 per cent of its tissues consisting of male germ cells is shown in Fig. 2, *E*. This gonad is in a mature condition, for mature ova are found in the medullary portion of the ovotestis near the beginning of the gonoduct instead of in the cortical region where ovocytes of various stages of development are seen. Empty spaces are left in the gonad apparently by the spermatozoa which have vacated the

FIG. 2. Drawings of different types of gonads of *Limacina retroversa*.

- A. Section of a complete gonad which is sexually undifferentiated.
- B. A "pure male" gonad in which no ovocytes are seen—section of a complete gonad.
- C. A hermaphroditic male gonad which has more than 75 per cent of its tissues made of male germ cells. Only the first or largest whorl is shown, the other two whorls being indicated by outlines.
- D. First or largest whorl of a hermaphroditic male gonad with 50–75 per cent of its tissues consisting of male germ cells.
- E. Right half of the first or largest whorl of a hermaphroditic female gonad which contains 25–50 per cent of male tissue.
- F. Right half of the first or largest whorl of a hermaphroditic female gonad with less than 25 per cent of its tissues made of male germ cells.

- 1. Nucleus of pregerminative cell.
- 2. Nucleus of spermatogonium.
- 3. Primary spermatocytes.
- 4. Secondary spermatocytes.
- 5. Spermatids.
- 6. Spermatozoa.
- 7. Ovocyte.
- 8. Ovum.
- 9. Outline of second whorl of ovotestis.
- 10. Outline of third whorl of ovotestis.
- 11. Outline of left half of first whorl of ovotestis.
- 12. Fibrous connective tissue surrounding gonad.
- 13. Retractor muscles.



gonad previously. Figure 2, *F* shows a gonad which is even more predominantly feminine than that shown in Fig. 2, *E*. Less than 25 per cent of this ovotestis is made up of masculine reproductive cells.

In contrast to masculine types, the pronounced feminine hermaphro-

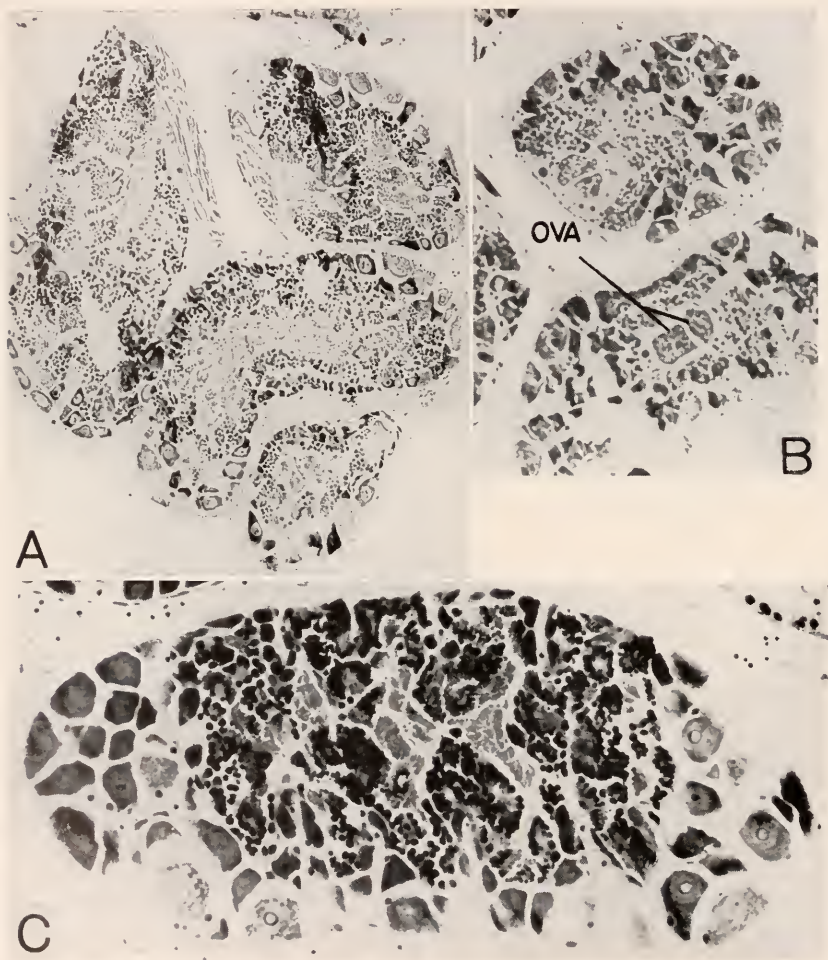


FIG. 3. Photomicrographs of sections of gonad of *Limacina retroversa*, showing hermaphroditic male and female gonads.

- A. Hermaphroditic male gonad in which more than 75 per cent of the tissues consists of male reproductive cells.
- B. Hermaphroditic female gonad in which 25-50 per cent of the gonadal tissues consists of male germ cells.
- C. Further enlarged view of a section of the second whorl of a hermaphroditic male gonad with 50-75 per cent male tissue, showing the germ cells in greater detail.

dites possessing very little male tissue are limited to medium and, more generally, to large-sized *Limacina*. Another difference between the extremely male and female types is that while "pure males" are common, forming about one-half of the protandric males, "pure females" have never been observed. All female *Limacina retroversa* are hermaphroditic.

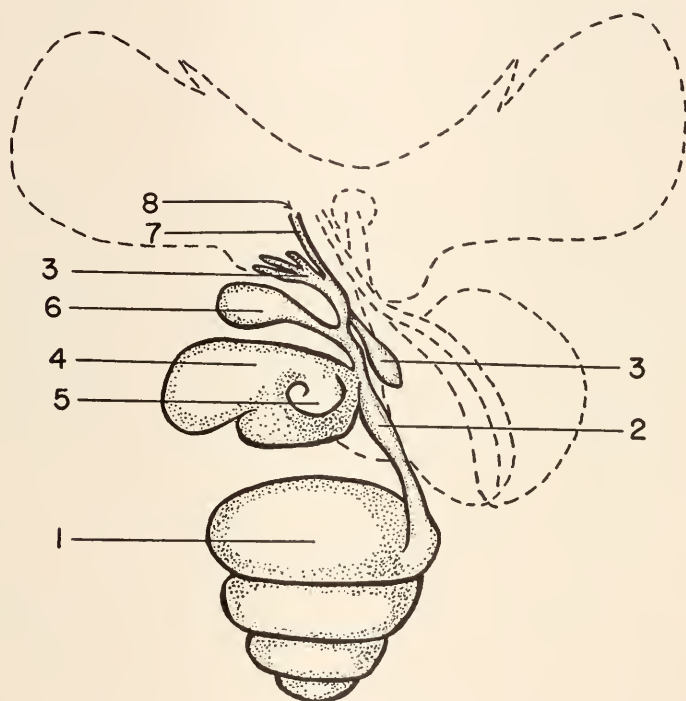


FIG. 4. Diagram of the reproductive system of *Limacina retroversa*.

1. Ovotestis.
2. Hermaphroditic duct or gonoduct.
3. Receptaculum seminis.
4. Shell gland.
5. Albumin gland.
6. Vesicula seminalis.
7. Out-going duct.
8. Genital opening.

Figure 3 shows some of the photomicrographs of sections of the gonad of *Limacina* upon which the diagrammatic drawings of Fig. 2 are based. Figure 3, *A* is a hermaphroditic male gonad with more than 75 per cent of its tissues consisting of male germ cells and Fig. 3, *B*, a hermaphroditic female gonad with 25–50 per cent of male tissue in it. Figure 3, *C* is a further enlarged view of a masculine gonad with

50–75 per cent male tissue showing the arrangement of the germ cells in greater detail.

The Accessory Organs of Reproduction.—The accessory organs of the reproductive system are diagrammatically represented in Fig. 4, which is a composite picture based upon the reconstruction from serial sections of the whole animal such as those shown in Fig. 5.

The gonoduct begins as a hermaphroditic duct for the passage of both ripe spermatozoa and ova. It opens into the ventral side of the anterior end of the spiral ovotestis and then turns to the right and passes forward, along the right side of the digestive track, to the anterior end of the body. It begins as a single tube with its wall composed of cuboidal cells, but later branches out into a pear-shaped structure (called "6" in Figs. 4 and 5). After meeting the accessory glands the gonoduct continues as the out-going duct. This part of the duct is much convoluted, leading to the external opening of the reproductive system on the right side of the mouth (8 in Fig. 4). This tube is composed of cuboidal epithelial cells with prominent nuclei and the cytoplasm of these cells contains a large quantity of secretory granules.

The accessory glands, like those of other pteropods, are very complicated structures. The largest gland, according to the terminology of Meisenheimer, is the shell gland. It is divided into three or four lobes occupying a great portion of the right side of the anterior part of the body of the animal. It opens into the hermaphroditic duct as well as the albumin gland (see Fig. 5, *D* and *E*). The shell gland is composed for the most part of large secretory cells, and tall, ciliated, columnar cells. The secretory cells are very abundant and obvious and with nuclei which are generally large and ellipsoidal, lying on the base of the cells away from the lumen of the gland, while the portion near it is filled with secretion. Perhaps the ovum stays longest in this gland on its way through the various parts of the reproductive system, for ova have been more often observed in this organ than in the others.

The albumin gland (Fig. 5, *B* to *D*) is a snail-like coiled gland associated with the shell gland but smaller than it. A diagram of its outline is shown in Fig. 4. The cells of the albumin gland are largely secretory. Their inner edge, which faces the lumen, is filled with transparent secretion when seen in sections stained with iron haematoxylin.

The vesicular seminalis (Figs. 4 and 5) is an elongated, pear-shaped organ connected with the hermaphroditic duct. It is lined with cuboidal epithelial cells. Inside this organ masses of mature spermatozoa are seen in the case of mature *Limacina*.

The receptaculum seminis is connected with the basal portion of the out-going duct. It consists of two portions. The first portion is much

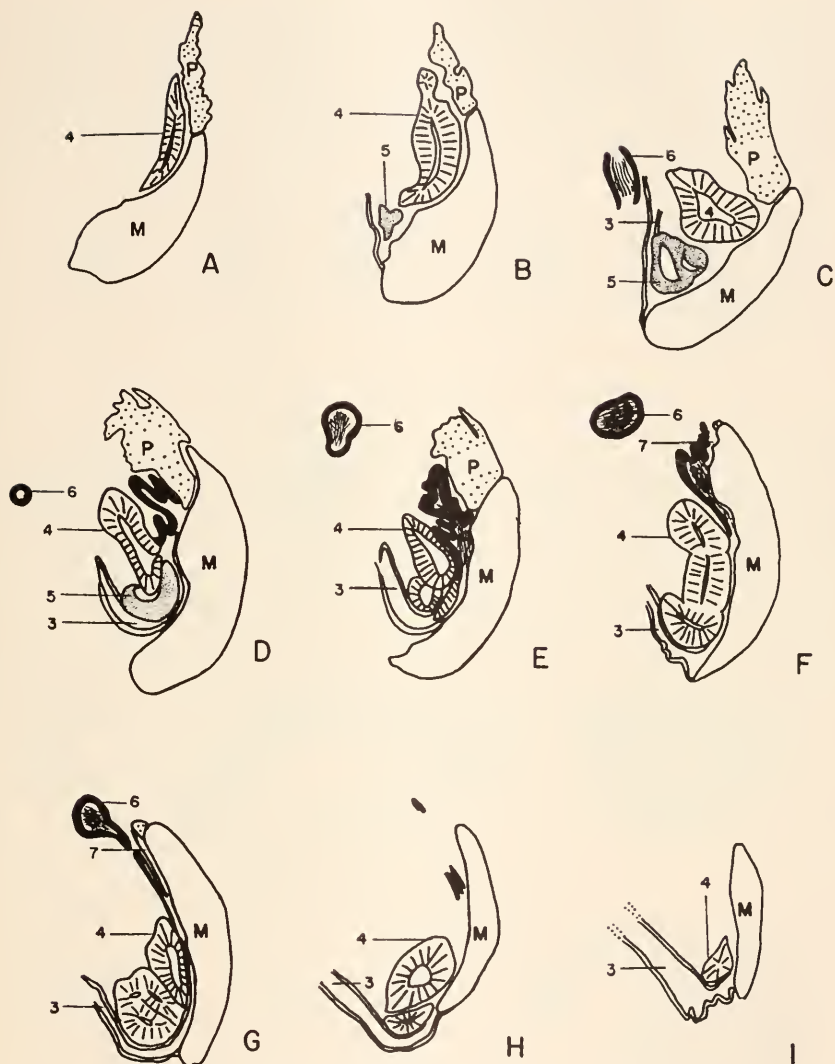


FIG. 5. Diagrams of longitudinal sections of the accessory organs of the reproductive system of *Limacina retroversa* (based upon camera lucida tracings from an animal cut into serial sections which are 5 micra thick).

Figure A. Section No. 90.

B. " " 100.

C. " " 115.

D. " " 124.

E. " " 135.

Figure F. Section No. 155.

G. " " 165.

H. " " 175.

I. " " 195.

M. Mantle gland.

P. Penis (in Figs. A-G).

3. Receptaculum seminis (Figs. C-I).

4. Shell gland (in all the figures).

5. Albumin gland (Figs. B-D).

6. Vesicula seminalis (Figs. C-G).

7. Out-going duct (Figs. F and G).

folded, with a thick wall composed of columnar cells, while the second portion is much thinner, being made of cuboidal cells and baggy in appearance (Figs. 4 and 5). Spermatozoa observed in the folded portion stain deeply with iron haematoxylin and are normal in structure, but those within the second portion stain only very lightly with the same

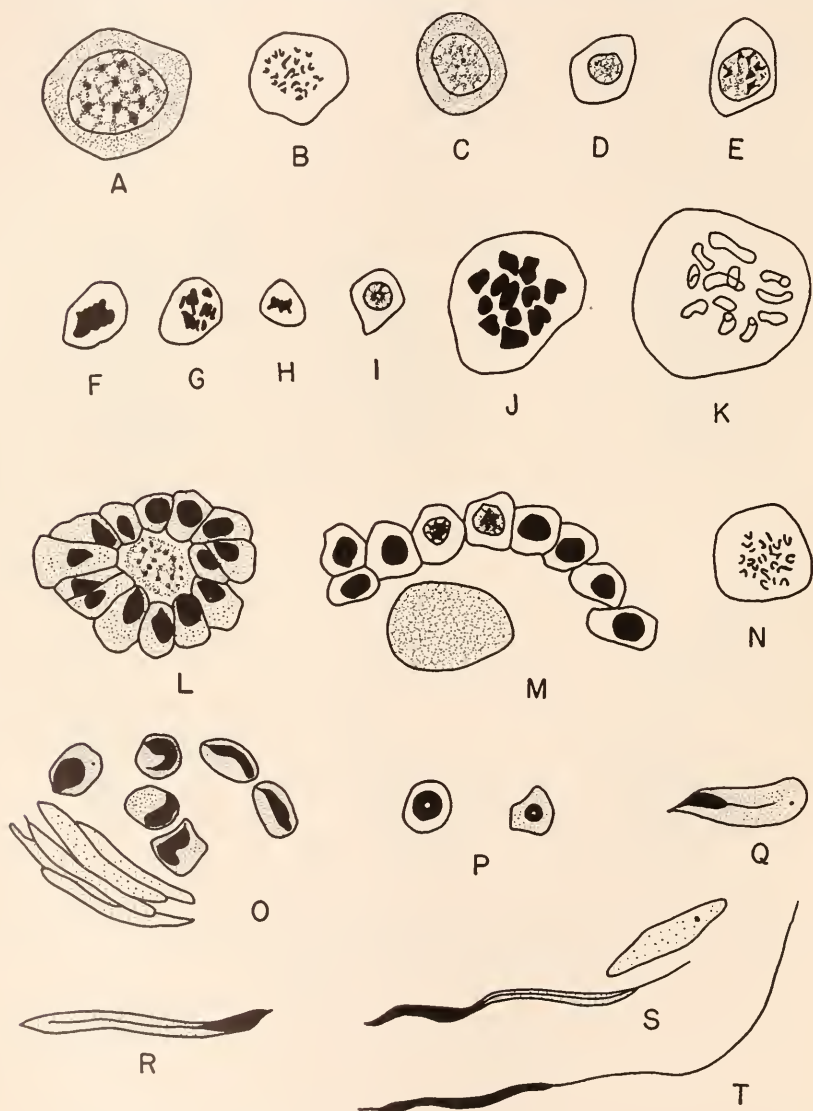


FIG. 6. Spermatogenesis of *Limacina retroversa*.

dye and show signs of degeneration and masses of disintegrating material are often observed.

The penis is practically independent of the rest of the reproductive system both in position and in structure. In well-developed adults it is a very large organ lying dorsad to the rest of the genital structures in the anterior portion of the body near the mantle gland (*P* in Fig. 5). This organ is very much folded on its outer surface when not distended. It has its own secretory glands and retractor muscles. The spermatozoa which come out of the gonoduct run along the ciliated seminal groove towards the penis along the right side of the dorsal region of the "fin" or "flapper."

GENERAL FEATURES OF GAMETOGENESIS

The development of the male and female reproductive cells has been examined from fixed material cut into serial sections and stained with iron haematoxylin as well as from fresh specimens treated with the carmine smear technique. The steps in spermatogenesis have been ascertained, and it has been found that the maturation of the male germ cells is essentially the same as that of other gastropods.

The pregerminative cells grow and multiply to form the primary spermatogonia (Fig. 6, *A*). The primary spermatogonia (Fig. 6, *B*)

EXPLANATION OF FIGURE 6

- A*. Primary spermatogonium.
- B*. Metaphase plate of primary spermatogonium showing the diploid number of 24 chromosomes.
- C*. Secondary spermatogonium.
- D*. Primary spermatocyte, beginning of leptotene threads.
- E*. Primary spermatocyte, showing tetrad condition.
- F*. Metaphase of first meiotic division, profile.
- G*. Polar view of anaphase of first meiotic division.
- H*. Early anaphase of second meiotic division, profile.
- I*. Young spermatid.
- J*. Chromosome plate of early anaphase of first meiotic division showing the haploid number of 12 chromosomes.
- K*. Chromosome plate of early anaphase of second meiotic division, showing 12 chromosomes.
- L*. A group of spermatids around a central cell.
- M*. Same as *L*, with spermatids not completely enclosing the central cell.
- N*. Metaphase plate of a somatic cell, showing the diploid number of 24 chromosomes.
- O*. Separate spermatids undergoing nuclear elongation, besides them a mass of discarded cytoplasm from other older spermatids.
- P*. Cross-sections of spermatids showing the central canal.
- Q*. Elongated spermatid, showing formation of tail filament.
- R*. Spermatid further elongated than *Q*.
- S*. Nearly mature spermatozoön with discarded cytoplasm beside it.
- T*. Mature spermatozoön.

divide by mitosis to give rise to the secondary spermatogonia (Fig. 6, *C*) which are smaller in size and with a higher nucleus-cytoplasm ratio than the primary. Generally the nucleus is placed on one side of the cell. Figure 6, *B* is a metaphase plate of a primary spermatogonium showing 24 chromosomes. The secondary spermatogonia differentiate into primary spermatocytes by mitosis. The primary spermatocytes are identified by the fact that they are nearly always grouped together in the gonad. At the first meiotic division the chromatin of the primary spermatocyte breaks up and forms loose masses and then changes into leptotene threads. These threads, as in some pulmonates, for example, *Succina ovalis*, *Ostrea lurida*, form such a compact mass that it is difficult to follow the steps in conjugation and tetrad formation (that is, parasynapsis and diakinesis). Figure 6, *D* shows the beginning of the leptotene threads with the nucleolus still visible and Fig. 6, *E* shows a protetrad condition of the chromatin material after parasynapsis. The tetrads of early diakinesis in the form of circles and crosses are arranged on the surface of the nuclear membrane and take a very dark color in staining. A profile of the metaphase of the first meiotic division is shown in Fig. 6, *F*, while Fig. 6, *G* is a polar view of the anaphase of the same division. Figure 6, *J* is a much enlarged polar view of the early anaphase of this division showing the reduced number of 12 chromosomes. During telophase the chromosomes draw together and form a compact mass in the secondary spermatocytes.

From the fact that a resting stage of the secondary spermatocyte is not seen it is inferred that this stage is most probably absent. This situation has been reported by Furrow (1935) for *Valvata tricarinata*. Cells undergoing the second meiotic division are smaller in size than those of the first. Figure 6, *H* is a profile of the early anaphase of the second meiotic division, and Fig. 6, *K* is a much enlarged view from one pole of the chromosome plate at an early anaphase of this division showing the haploid number of twelve chromosomes. During the telophase of this division the chromatin material becomes reticular as the nuclear membrane reappears and later condenses into a very dark staining structure which eventually becomes the dark nucleus of the spermatid. In Fig. 6, *I* a newly-formed nucleus of a younger spermatid is shown. Two of the spermatids of Fig. 6, *M* are also in this condition. These figures show the formation of reticular structures within the nuclear membrane and the condensation of the chromatin material in forming the dense nucleus of the spermatid.

The spermatids are generally arranged around a large degenerating cell which may be homologized with the "nurse cell" reported in most mollusks, but no cytoplasmic connection between it and the spermatids

has been observed (Fig. 6, *L* and *M*). In spermiogenesis the darkly-staining nucleus elongates first at one point so as to assume an acutely ovate outline (Fig. 6, *L*). A few of the various shapes assumed by the nucleus during its metamorphosis into the head of the mature spermatozoön are shown in Fig. 6, *O*. In association with the change of the nucleus, the cytoplasm elongated and the axial filament of the tail-piece appears (Fig. 6, *Q*). In the cross-section of an old spermatid of this stage the central canal through the nucleus can be seen as a light area in the center (Fig. 6, *P*). The spermatid is transformed into a mature spermatozoön by further elongation of the cytoplasm together with the growth of the axial filament and lengthening of the nucleus followed by a discarding of a greater portion of the cytoplasm (Fig. 6, *Q* to *S*). The head-piece of the spermatozoa is slender and pointed at the anterior end. It is formed by the nucleus. The tail-piece consists of an axial filament and a very slender tail membrane formed by the cytoplasm. The middle piece of the spermatozoön is not clearly seen and its development could not be followed in our material. A mature spermatozoön (Fig. 6, *T*) is about 30 micra long and its head about half as long as its tail-piece. During spermiogenesis the spermatids move away from a spherical arrangement about a central "nurse cell" and become irregularly placed as seen by a comparison of Fig. 6, *L* and Fig. 6, *O*. When the spermatozoa are mature they are placed closely together side by side with their heads pointing in the same direction, but they do not form "sperm balls" or "sperm morulae." In no case has a "nurse cell" been observed to which the heads of the spermatozoa are attached as has been reported for some pulmonates and prosobranchiates.

In specimens used in this study no atypical spermatogenesis has been observed. The only element in the gonad which might suggest apyrene spermatozoa is that shown in Fig. 6, *O*. It consists of a mass of elongated, enucleated structures which stain very lightly. But it appears more probable that they are discarded portions of the cytoplasm of the transforming spermatids rather than apyrene spermatozoa for no steps comparable to the atypical spermatogenesis leading to the formation of these structures have been observed.

The diploid number of chromosomes is 24. This number was first deduced from observations on the secondary spermatocytes which exhibit the haploid number of 12 chromosomes and was again checked against the chromosome plates of somatic cells. A metaphase chromosome plate of a somatic cell drawn with a camera lucida from a carmine smear is shown in Fig. 6, *N* and it may be compared with the chromosome plate of the mitotic division of the spermatogonium shown in Fig. 6, *B*. The chromosome number was further confirmed by the chromo-

some plates of the second mitotic division which also shows (Fig. 6, *K*) the haploid number of 12 chromosomes.

It has been observed in young gonads (Fig. 2, *B*) that there were at first only a few clusters of spermatozoa in the whole organ. As the animal grows the number of clusters of spermatozoa increases and eventually, in a mature *Limacina*, the mass of spermatozoa constitutes nearly all the male tissue in the gonad. It has been reported in other mollusks that each cluster of spermatozoa descends from one primary spermatogonium. If this is also the case in *Limacina*, then it appears from this gradual increase of the number of clusters of spermatozoa with the

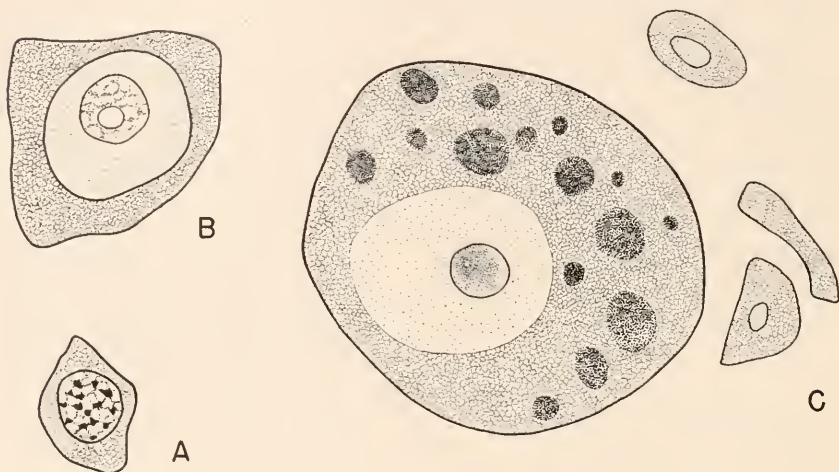


FIG. 7. Drawings of the female reproductive cells of *Limacina retroversa* (Fleming).

A. An oögonium.

B. A growing ovocyte with homogeneous cytoplasm.

C. A nearly mature ovum with a number of darkly-staining granules in the cytoplasm.

growth of the animal that the spermatogonia do not all develop at the same time, but that they follow each other in their maturation.

The female cells as a whole evidently develop slower than the male so that the animal generally assumes a protandric condition. The process of maturation of the female reproductive cells cannot be readily observed in our specimens of *Limacina retroversa*. When the different ovocytes in the ovotestis are compared it is seen that the growth of the cells is associated with the deposition in the cytoplasm of large granules which stain very dark with haematoxylin. The nucleus, on the other hand, takes stain less and less easily and eventually becomes much lighter as the ovocytes grow in size. A mature ovum measures 50 to 70 micra,

which represents an increase of five to seven times in diameter over the first ovocyte. Figure 7 shows an oögonium together with a growing ovocyte and a nearly mature ovum. The change in size is quite noticeable.

As in the case of spermatogenesis, the ova do not all mature simultaneously, but only a few are fully developed at a time. When once started, oögenesis seems to continue throughout the remainder of life of the individual. Female germ cells before and during maturation are located on the peripheral region of the gonad, but ova, when mature, migrate to the central portion of the gonad and thence to the opening of the gonoduct (Figs. 2, *D* and 2, *E*). Fertilization probably takes place during the passage of the ova through the gonoduct.

Female reproductive cells of different ages are present in the gonad, but the maximal number of mature ova observed in a single five micra section of one whorl of the ovotestis is not greater than ten to twenty. The largest number of ova observed in the gonoduct at the time of fixation is about four or five. These observations, together with the fact that *Limacina retroversa* kept in the laboratory lay a few eggs at a time, indicate that this animal does not spawn out after a brief period of intensive reproductive activity, but that spawning is more or less protracted and continuous after it has once begun.

DISCUSSION AND SUMMARY

From the above description of the maturation of the germ cells of *Limacina retroversa* it will be seen that the process of spermatogenesis in this species agrees with those of other gastropod mollusks. Von Brunn (1884) first observed atypical spermatozoa and considered them to be rudimentary ova. Meves (1903) and Hertwig (1905), however, have described the steps in atypical spermatogenesis and tried to elucidate the morphological and physiological significance of this process and its product. Reinke (1914) in *Strobus bituberculatus* and Kuschakewitsch (1910, 1911) in *Conus* and *Vermetus*, Gould (1917) in *Crepidula plana*, and other authors, made similar studies. Recently Hickmann (1931) described atypical spermatogenesis in *Succina ovalis*, Furrow (1935) in *Valvata tricarinata*, Coe and Turner (1938) in *Mya arenaria*. But as far as it can be ascertained there is no atypical spermatogenesis in *Limacina retroversa*. This species seems to belong to the group of mollusks that do not show atypical spermatogenesis.

Nurse-cells have been reported in many forms of mollusks during their maturation, but in *Limacina retroversa* the arrangement of the spermatids and newly matured spermatozoa about a central cell may

suggest the presence of nurse-cells though no cytological connection has been observed between these and the germ cells.

Mature spermatozoa form balls or "sperm morulae" (according to Orton, 1926) in certain mollusks while in others, though of the same genus, they do not. Thus, *Ostrea lurida* and *Ostrea virginica*, in this country, have clustered and separate mature spermatozoa respectively. *Limacina retroversa* have separate mature spermatozoa.

The accessory organs of the reproductive system of *Limacina retroversa* agree very closely with the description given by Meisenheimer, who did not mention which species were used as representatives of the genus *Limacina* in his comparative studies. However, if Meisenheimer's work on the secondary sexual organs is taken as a typical description of the genus *Limacina*, then *Limacina retroversa* may be considered as closely resembling the typical forms in the structures of the accessory sexual organs.

BIBLIOGRAPHY

- BIGELOW, H. B., 1926. Plankton of the off-shore waters of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40** (Part II): 1 (1924).
- BONNEVIE, K., 1916. Mitteilung über Pteropoden. L. Beobachtungen über den Geschlechtsapparat von Cuvierina columnella Rang. *Jena Zeitschr. Nat.*, **54**: 245.
- BOURNE, G. C., 1889. The generative organs of the oyster. (Abstract of a paper by Dr. P. P. C. Hoek.) *Jour. Mar. Biol. Ass'n*, **1**: 268.
- COE, W. R., 1931. Spermatogenesis in the California oyster (*O. lurida*). *Biol. Bull.*, **61**: 309.
- COE, W. R., 1933. Sexual phases in *Teredo*. *Biol. Bull.*, **65**: 283.
- COE, W. R., AND H. J. TURNER, JR., 1938. Development of the gonads and gametes in the soft-shell clam (*Mya arenaria*). *Jour. Morph.*, **62**: 91.
- FLEMING, J., 1823. On a reversed species of *Fusus* (*Fusus retroversus*). *Mcm. Werner. Soc.*, **4**: 498.
- FOL, H., 1874. Note sur le développement des Mollusques Ptéropodes et Cephalopodes. *Arch. d. Zool. Expér. et Gén.*, **3**: 33.
- FOL, H., 1875. Études sur le développement des mollusques. I. Sur le développement des ptéropodes. *Arch. Zool. Expér. et Gén.*, **4**: 1.
- FURROW, C. L., 1935. Development of the hermaphrodite genital organs of *Valvata tricarinata*. *Zeitschr. f. Zellforschung.*, **22**: 282.
- GEGENBAUR, C., 1853. Bau der Heteropoden u. Peteropoden. *Zeitschr. f. wiss. Zool.*, **4**: 334; 369.
- GEGENBAUR, C., 1855. Untersuchungen über Pteropoden u. Heteropoden. Ein Beitrag zur Anatomie u. Entwicklungsgeschichte dieser Thiere. Leipzig.
- GOULD, H. N., 1917. Studies on sex in the hermaphrodite mollusc (*Crepidula*). I. History of sex cycle. *Jour. Exper. Zool.*, **23**: 1.
- HERTWIG, R., 1905. Über das Problem der sexuellen Differenzierung. *Verh. deutsch. Zool. Ges.*, **15**: 186.
- HICKMAN, C. P., 1931. Spermatogenesis of *Succinea ovalis* Say, with special reference to the components of the sperm. *Jour. Morph. and Physiol.*, **51**: 243.
- KNIPOWITSCH, N., 1891. Zur Entwicklungsgeschichte von *Clione limacina*. *Biol. Centralbl.*, **11**: 300.

- KROHN, A., 1856. Beobachtungen aus der Entwicklungsgeschichte der Pteropoden, Heteropoden und Echinodermen. *Müller's Arch. f. Anat.*, p. 515.
- KUSCHAKEWITSCH, S., 1910. Zur Kenntnis der sogenannten warmförmigen spermien der Prosobranchier. *Anat. Anz.*, **37**: 318.
- KUSCHAKEWITSCH, S., 1911. Über die Entwicklung der spermien bei *Conus mediterraneus* Brug. und *vermatus gigas* Biv. *Biol. Centralbl.*, **31**: 530.
- LOOSANOFF, V. L., 1937. Development of the primary gonad and sexual phases in *Venus mercenaria* L. *Biol. Bull.*, **72**: 389.
- MEISENHEIMER, J., 1905. Pteropoda. *Wiss. Ergebn. de Deutsch. Tiefsee Exp., Valdivia*, **9**.
- MEVES, F., 1903. Ueber oligopyrene und apyrene spermien und ihre Entstehung nach Beobachtungen an *Paludina* und *Pygaera*. *Arch. f. micros. Anat.*, **61**: 1.
- MÜLLER, J., 1858. Sur le développement des Pteropoden. *L'Institute*, **26**: 37.
- ORTON, J. H., 1909. On the occurrence of protandric hermaphroditism in the mollusc *Crepidula fornicata*. *Proc. Roy. Soc., B*, **81**: 468.
- ORTON, J. H., 1926. Observations and experiments on sex-change in the European oyster (*O. edulis*). *Jour. Mar. Biol. Ass'n*, **14**: 967.
- REINKE, E. E., 1912. A preliminary account of the development of the apyrene spermatozoa in *Strombus* and of the nurse-cells in *Littorina*. *Biol. Bull.*, **22**: 319.
- REINKE, E. E., 1914. The development of apyrene spermatozoa of *Strombus bituberculatus*. *Carnegie Inst. Washington*, Publ. No. 183, p. 195.
- SOULEYET, L., 1843. Observations anatomiques, physiologiques et zoologiques sur les mollusques ptéropodes. *Compt. Rend. Acad. Sci.*, **17**: 662.
- STEPHAN, P., 1903. Sur les spermies oligopyrènes et apyrènes de quelques Prosobranches. *Compt. Rend. Soc. Biol., Paris*, **55**: 554.
- TESCH, J. J., 1913. Pteropoda. *Das Tierreich*, **36**.
- THIELE, J., 1931. Handbuch der Systematischen Weichtierkunde.
- VAN BENEDEN, P. J., 1839. Memoire sur la *Limacina arctica*. *Nouv. Mém. Acad. Roy. Sci. et Belles Lettres de Bruxelles*, T. 14.
- VON BRUNN, M., 1884. Untersuchungen über die doppelte Form der Samenkörper von *Paludina vivipara*. *Arch. Mikrosk. Anat.*, **23**: 413.
- ZARNIK, B., 1911. Über den Chromosomencyclus bei Pteropoden. *Verh. deutsch. Zool. Ges., Leipzig*, **20**: 205.

THE HISTORY OF A POPULATION OF LIMACINA RETROVERSA DURING ITS DRIFT ACROSS THE GULF OF MAINE

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Geographical distribution in the sea is a hydrodynamic problem, in which the flow of waters determines not only the "climate" of specific regions, but also the supply of organisms to populate these regions. A pelagic population must drift with the waters in which it lives. In contrast to littoral and terrestrial populations, there is no inherent fixity in its occurrence, save that determined by the current systems of the sea. The continuous drift of water through a given region not only raises questions regarding the maintenance of a population there (Damas, 1905; Sømme, 1933, 1934); it renders continuous observation on the natural history of the population singularly difficult, since the individuals observed at any place one day are different from those observed at the same place on another occasion.

Immigrant plankton which do not thrive or reproduce in the Gulf of Maine, but which are carried into its waters from offshore, were found by Bigelow (1926) to occur in a zone extending from the Eastern Channel along the eastern, northern and western sectors of the Gulf as though carried thither by the great cyclonic eddy which Huntsman (1924) and Bigelow (1927) have shown to dominate the circulation of the Gulf. Animals of arctic origin, *Limacina helicina*, *Mertensia ozum*, *Oikopleura van hoffeni*, and *Ptychogena lactea* were taken only in the eastern sector of this zone, apparently being unable to survive long enough to be borne further. Other immigrants, both of boreal and tropical origin, survived until carried as far as the offing of Cape Cod, but few were ever taken in the southern sector of the circuit. As his cruises were not taken with sufficient frequency, these observations yield no evidence concerning the velocity of the drift on which these invaders were borne, although its course was clearly enough indicated.

The observations on the pteropod *Limacina retroversa* Fleming² de-

¹ Contribution No. 196 from the Woods Hole Oceanographic Institution.

² We have employed the name *Limacina retroversa* Fleming because of its current use in oceanography. Dr. W. J. Clench advises me that the generic name

scribed in this paper are of interest because during the greater part of the year it has been possible to follow the history of a rather definite population as it drifted across the Gulf of Maine, noting its rate of spread, its mortality, the growth and reproduction of its individuals, and its replacement by another population of the same species.

Limacina retroversa is a boreal form occurring from latitude 50° to northern Norway off the European coast and from latitude 34° to the southern part of Davis Strait in the western Atlantic. Bigelow considered it one of the most characteristic of the permanent pelagic inhabitants of the Gulf of Maine "where its numbers depend on local reproduction and not on immigration from elsewhere." He also emphasizes the irregularity of its occurrence, an opinion with which I can agree since my observations are at some variance with his. The seasonal variation in the distribution of *Limacina* shown in Fig. 44 of Bigelow's monograph led me to suspect that its occurrence might be closely correlated with the residual drift of the superficial water, since the captures in December and January extend along the northern and western shores of the Gulf, those from February to April occur over the western and southern sectors, while records for May extend from this zone northeasterly toward the Bay of Fundy.

During the years 1933-1934 a systematic survey was made of the Gulf of Maine with the object of obtaining a more precise seasonal picture of its hydrography and biology than was available from the observations of Bigelow. Thirteen cruises were made by the research vessel "Atlantis" in the course of fifteen months, with the result that 684 hydrographic stations in the Gulf of Maine and its adjacent waters were occupied. Whenever weather permitted, a standard vertical haul was made at each station using a Heligoland larva net similar to that described by Künne (1933). The opening of the net was 143 cm. in diameter and it was made of No. 0 silk having 38 meshes to the inch. The net was drawn from a point near the bottom to the surface. The catch of each net haul was preserved and from it the *Limacina* have been separated, counted and measured.

Seasonal Distribution and Abundance

The seasonal distribution and abundance of *Limacina* throughout the year, as shown by the number of specimens caught in each haul, is presented in Figs. 1 and 2. *Limacina* was scarce in the Gulf of Maine

Spiratella has claims of priority arising from Blainville's description (*Dict. Sci. Nat.*, ix, 407, 1817). No attempt has been made to distinguish *L. retroversa* from *L. balea* Möller since the distinctness of these species is in doubt (Bigelow, 1926, p. 116).

in the fall of 1933. A dense population of these organisms appeared in the eastern side of the Gulf in December, 1933 and spread westward during the winter. By April these pteropods are concentrated in the western side of the Gulf; the region to the eastward in which they first appeared being occupied by waters scantily populated. As the season advanced the size of the catch at each haul dwindled and the distribution became more general.

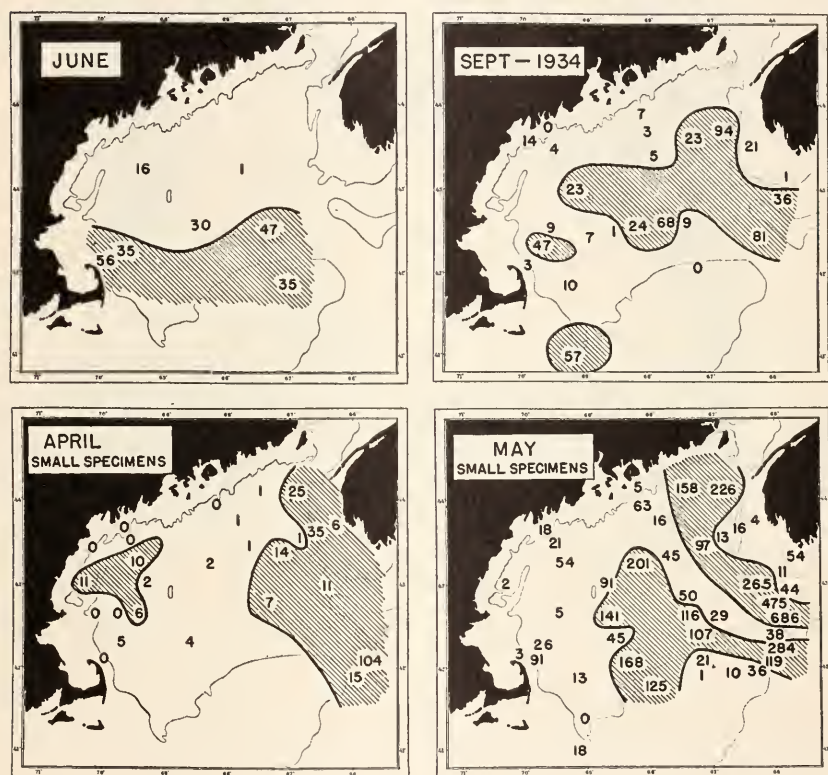


FIG. 2. Charts showing the distribution of *Limacina retroversa* of all sizes in the Gulf of Maine in June and September, 1934, and of small individuals attributable to population B in April and May, 1934.

These observations suggest that a huge swarm of *Limacina* drifted into the Gulf from the east in December and were carried westward by the cyclonic drift of water about the Gulf. The acceptance of this interpretation requires evidence on two points: (1) that the population moved not only in the direction but at a rate corresponding to the mass movement of the water, and (2) that a reasonable continuity exists between

the population observed in December in the eastern region and that found in April and May in the western part of the Gulf.

The Circulation of the Gulf

The character of the circulation of the Gulf is adequately described by Bigelow (1927), who with Huntsman showed that the dominant circulation is a great anti-clockwise eddy around the basin of the Gulf,

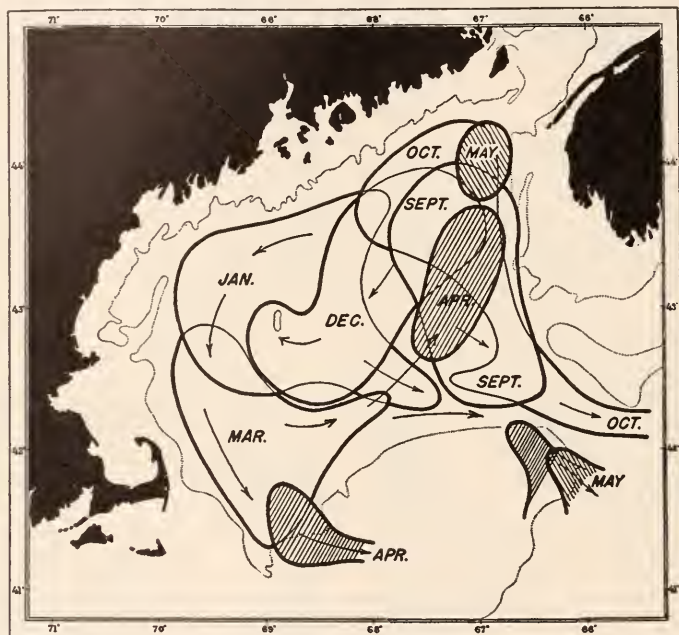


FIG. 3. Positions occupied by an area in which the salinity of the water at 50 meters depth exceeded 33.0‰ during the period from September, 1933, to May, 1934. Arrows indicate the apparent direction of drift at each period.

varying in velocity and in detail from season to season and complicated by subsidiary eddies. The great eddy receives accessions of water from the east, particularly in winter and early spring; the area of inflow being over the Northern Channel and the adjoining banks. Compensatory losses are occasioned by outflow across the Southern Channel and around the eastern end of Georges Bank. The circulation at the greater depths has a somewhat modified character owing to the confining features of the bottom contour. A report on the dynamics of the circulation of the Gulf, based on observations made during the cruises of 1933-34, is being prepared by Dr. E. E. Watson.

While our vertical hauls have given no indication of the depths at which *Limacina* occurs, it seems probable that it will be carried with the more superficial circulation of the Gulf. Bigelow (1926, p. 121) states that "the most prolific depth zone may be stated as from 20 to 25 meters down to about 80, which corroborates Paulsen's (1910) generaliza-

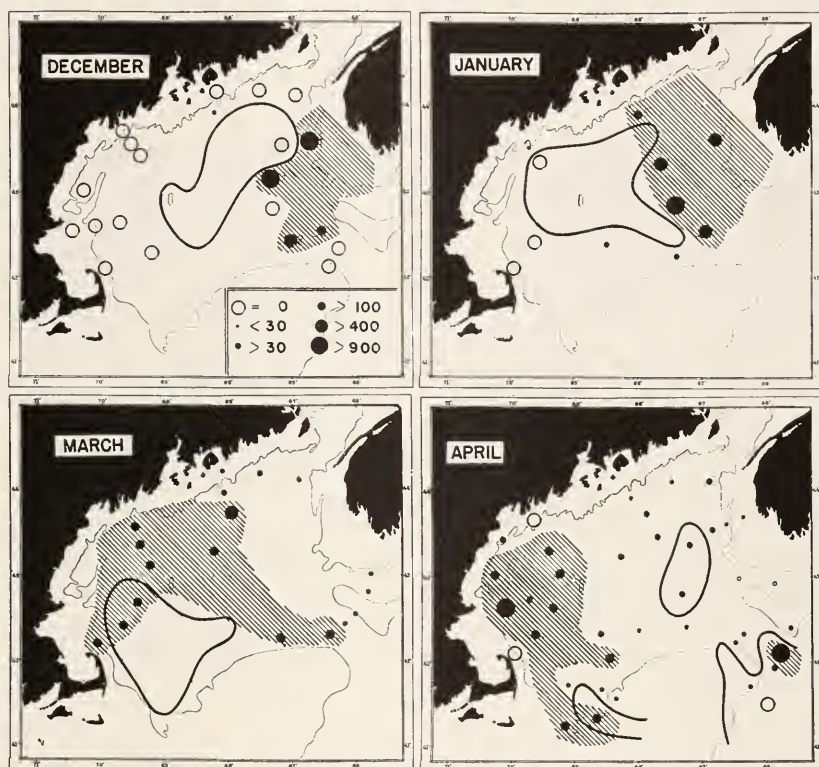


FIG. 4. Charts showing the relative positions of the area of high salinity at 50 meters depth and the distribution of *Limacina* between December, 1933 and April, 1934. The closed contours enclose the area in which $S > 33\text{‰}$. The hatched areas include catches greater than 100 specimens per haul. The size of each catch is roughly proportional to the area of the black circles. Open circles represent hauls in which no *Limacina* were caught.

tion that *Limacina* lives chiefly shoaler than 50 meters in north European waters, though it has occasionally been taken much deeper." That the sub-surface waters actually drift with velocity as well as direction comparable to that characterizing the *Limacina* population is indicated by a study of the distribution of salinity in the upper water levels throughout the period of the survey. An extensive area characterized

by salinities higher than the surroundings occurred in the eastern part of the Gulf in September, 1933. Bigelow (1927, pp. 767, 768) noted a similar occurrence in 1913, 1914 and 1915. The position of this area gradually shifted during the following months, until March when it occurred in the southwestern part of the Gulf. Subsequently it could be traced with less certain continuity as it divided, apparently drifting in part out to sea, and in part in a northeasterly direction. The "saline pool" is most clearly limited in the data for salinities at 50 meters, at which depths it is enclosed by the $33^0/_{00}$ isohaline. The successive

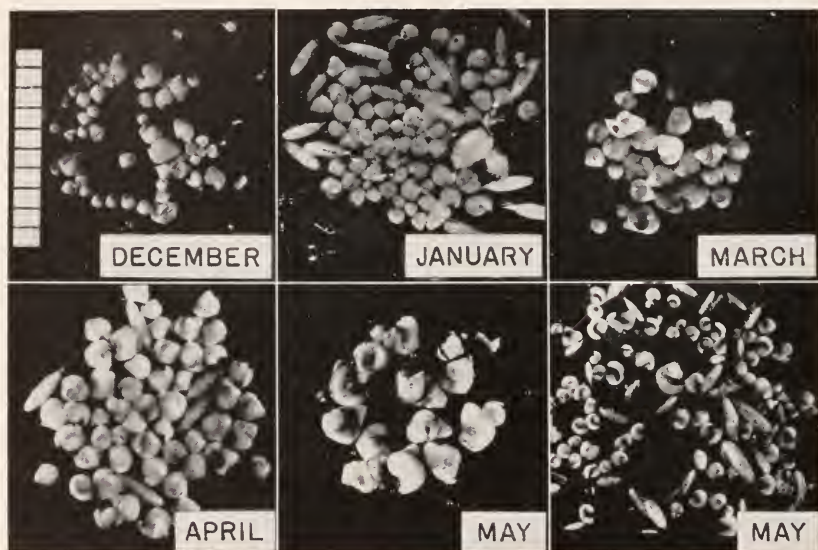


FIG. 5. Photographs of representative catches of *Limacina retroversa* taken between December, 1933 and May, 1934 showing growth. The large specimens taken in May are from the western basin and represent the size attained by population A. The small May specimens were taken in the eastern part of the Gulf and are attributed to population B. Scale, 1 centimeter divided into tenths.

positions of this isohaline are illustrated in Fig. 3, and correspond closely to the superficial circulation as deduced and illustrated by Bigelow (1927). The velocity of drift of this area, characterized by relatively high salinity, corresponds closely to that of the *Limacina* population which apparently followed close in its rear (Fig. 4). The observations on the distribution of *Limacina* and the occurrence of water of $S > 33^0/_{00}$ at 50 meters depth agree in indicating a drift during the winter period of the sub-surface waters through a distance of 150 miles in four months or at a rate of about 1.25 miles per day.

The Distribution of Size at Different Seasons

The identity of the population sampled at different periods is indicated by an examination of the distribution of size among the specimens taken. These appeared to grow larger as the winter advanced (see Fig. 5). In April and May, however, large numbers of small specimens appeared in certain catches. Since the animals taken at different times and places can only be attributed to the same population if they display a reasonable relation in size, the distribution of size among the individuals of each catch was determined. The largest diameter of the specimens was measured as they lay in random positions in a watchglass. Care was taken to avoid measurements along diameters exaggerated by the spreading of the foot as it protruded from the shell. The measurements were separated into 9 size groups of 0.3 mm. span. The number in each group was expressed as a percentage of the total catch falling within each group.

Figure 6 shows the distribution of size among all the specimens taken during each cruise. In December size distribution is homogeneous; the modal class size is 0.6–0.9 mm. with a uniform distribution of smaller and larger sizes ranging between 0.3 and 1.2 mm. In January the modal class size is the same, but the larger classes have grown at the expense of the smaller. By March a general increase in size is apparent, the modal class being 1.2–1.5 mm. and almost all specimens falling between 0.6 and 1.8 mm. The general shape of the polygon is unchanged as should be the case if the increase in size is due to uniform growth. In April the frequency polygon becomes skewed. Though the modal size is the same as in March, the larger classes have increased in number, relative to the mode, as demanded by growth. The skew is due to the appearance of significant numbers of very small (less than 0.6 mm.) individuals which were not present in the March population. In May these small specimens increase greatly in numbers with the result that the frequency polygon is bimodal. The principal modal size is now 0.3–0.6 mm., being determined by the small individuals. A secondary mode occurs between 1.5 and 1.8 mm., apparently representing the mode of the original population which determined the mode during the previous periods.

The observations made between December and May may be readily interpreted as follows. A homogeneous population of small individuals entered the Gulf from the east in December and grew continuously in size. In April a new population of small individuals appeared and by May these had increased in numbers so as to dominate the size distribution. These may represent a generation of offspring from the original

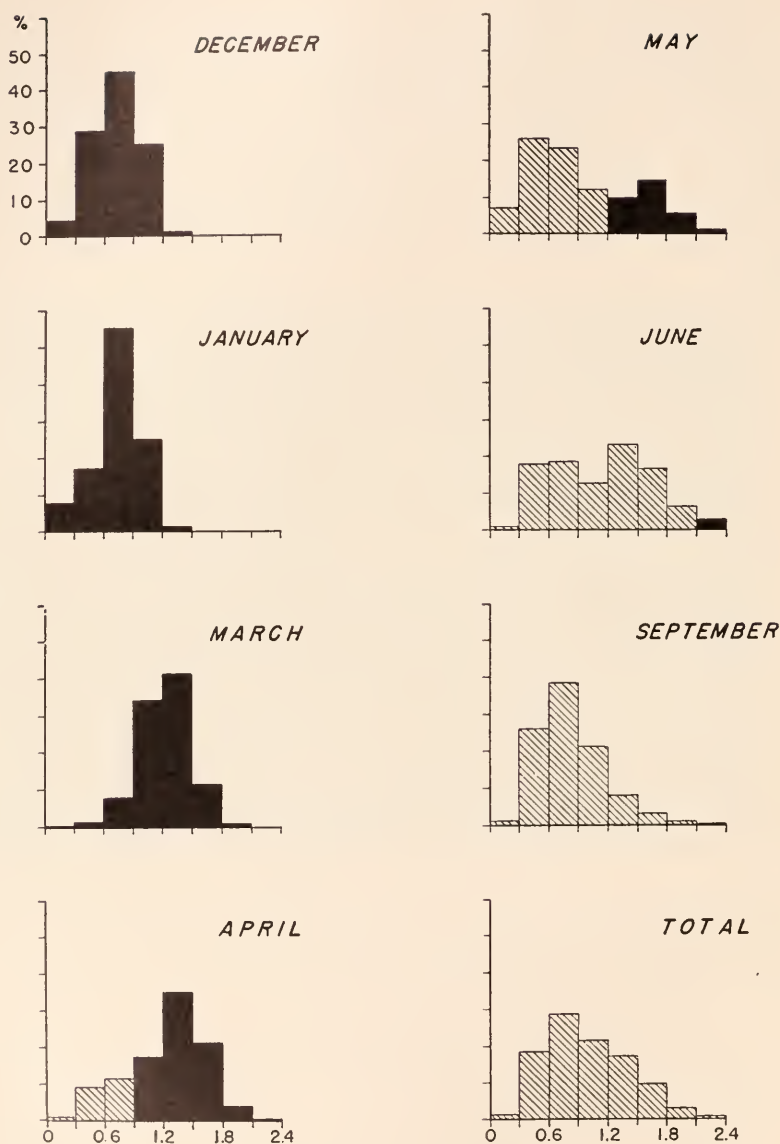


FIG. 6. Frequency polygons showing the distribution of size in the total catch of *Limacina* obtained during each cruise. The lower right-hand polygon represents the combined distribution for the entire series of cruises, corrected for variation in fishing effort. Ordinates, percentage of entire catch falling in each size group. Abscissae, size groups divided in intervals of 0.3 mm. diameter. Black areas are attributed to population A.

population, or an invasion of a new group from outside the Gulf, or both. For convenience we will call the original population which appeared in December the *A* population; the new population which appeared in April the *B* population.

The observations made subsequent to May are more difficult to interpret. The frequency polygon for June is again bimodal. The principal size class is now 1.2–1.5 mm. This is smaller than the mode of the *A* population for the preceding month and may be tentatively assigned to the *B* population, which may be expected to grow rapidly with the warming of the water. The largest size class, 2.1–2.4 mm., is relatively more abundant than at any previous time and probably contains the last survivors of the *A* population. A secondary mode occurs between 0.3 and 0.9 mm., which may represent a further production or a new invasion of young individuals, a hypothetical *C* population. The small specimens taken in June were caught a few miles off the coast of Cape Cod. Because of the remoteness of this position from the inflow into the Gulf, they are in all likelihood the offspring of the *A* population, which was richly represented in this region during the preceding cruise, rather than to a new invasion. Only five hauls were made during the June cruise and these all in the southwestern half of the Gulf. The data for this period are not as representative as those obtained at other times.

The scanty and scattered catches made in September, 1934 yield a simple frequency polygon with maximal size class at 0.6–0.9 mm. The distribution of size is generalized and resembles that of the combined measurements of the total collections throughout the year. Apparently during the summer growth is rapid and reproduction and recruitment of the population from without the Gulf is more or less continuous so that separate broods or populations can no longer be distinguished. Dr. Sidney Hsiao has studied the reproductive history of the collections and finds that *Limacina* produce eggs in small numbers continuously after reaching a size of 1.0 mm. It may be inferred that the offspring of one generation will vary greatly in age and after several generations their descendants may be represented by a complete assortment of sizes in which separate broods will be indistinguishable.

The Distribution of Size in the Population of Different Sectors of the Gulf

Let us return to the examination of the history of populations *A* and *B* with a view to determining whether they are actually homogeneous populations and to distinguishing whether population *B* arises through reproduction within the Gulf, or invasion from outside.

The size distribution of representative catches made in different parts of the Gulf at each period has been examined. For this purpose the Gulf has been divided into seven sectors as indicated in Fig. 7. These sectors are arranged in the order in which the non-tidal drift of water may be expected to carry a pelagic population in the circuit of the Gulf. It should be noted, however, that much water will be carried from the



FIG. 7. Chart of Gulf of Maine showing principal place names and the sectors into which the area is divided for analysis of population distribution. Contour encloses depths less than 100 meters.

Yarmouth and Mt. Desert sectors directly to the Georges sector without penetrating the western sectors of the Gulf, as Fig. 3 indicates. Representative catches made at each period in each of these sectors have been selected and their size frequency polygons are given in Fig. 8.

Figure 8 shows that from December through March the size distribution in different parts of the Gulf is homogeneous and that the in-

crease in size noticeable in March is of general occurrence. The extension of the population westward during the winter is apparent. The

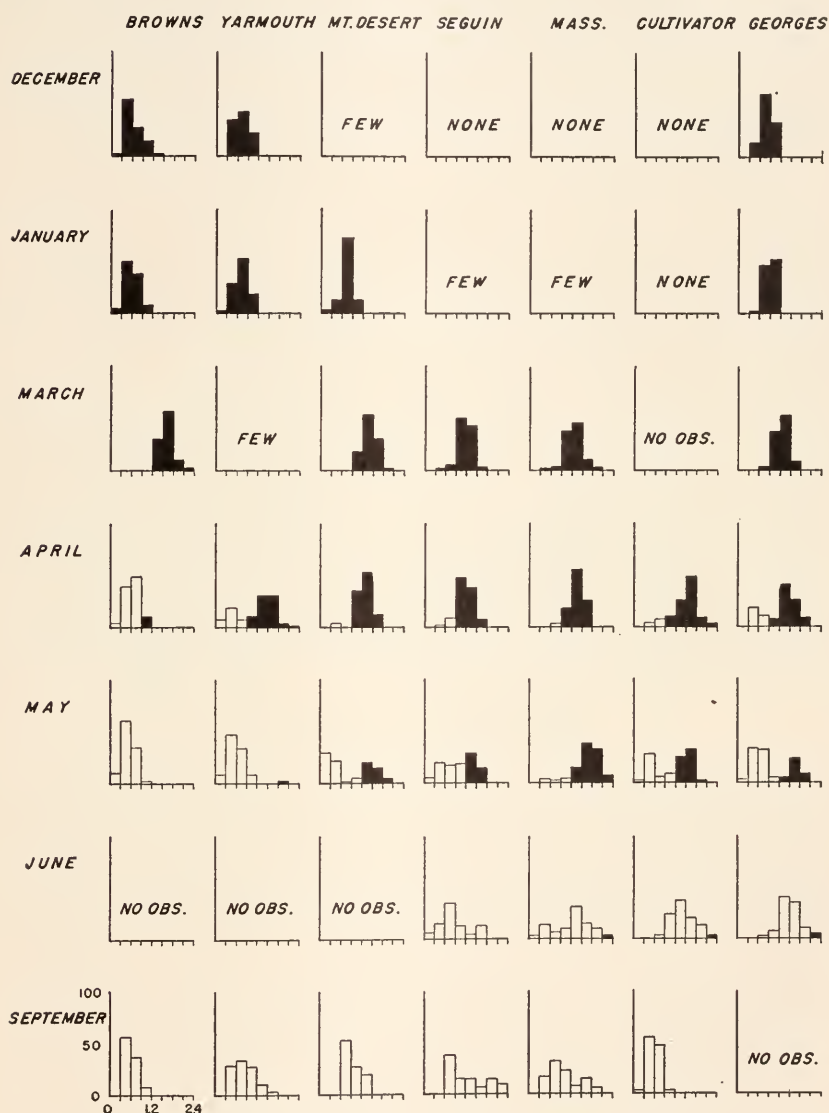


FIG. 8. Frequency polygons showing the distribution of size in representative catches of *Limacina* from each sector of the Gulf of Maine during each cruise. No hauls were made in the sectors marked *No Obs.* Ordinates, percentage of entire catch falling in each size group. Abscissa, size groups divided in intervals of 0.3 mm. diameter. Black areas are attributed to population A.

continuous occurrence of *Limacina* in the Georges sector is in accordance with the characteristics of the current system as noted above. In April the homogeneous *A* population appears in all the northern and western sectors of the Gulf. Small individuals representing the *B* population appear now in the eastern sectors, Browns, Yarmouth and Georges. In the outermost sector to the east, Browns, the *B* population is unaccompanied by any specimens of larger size, characteristic of the *A* population at this time.

In May the small individuals of the *B* population are much more widespread, appearing abundantly in all sectors except the most westerly, Massachusetts sector. Only small individuals occur in the stations of the Browns and Yarmouth sector. The populations of the Mt. Desert, Cultivator and Georges sectors have a mixed, bimodal character. The duplex character of the population in April or May is brought out in Figs. 1 and 2, in which the numerical distribution of individuals of different sizes is shown. Small specimens appear in numbers only in the water in the offing of the Eastern Channel in April and occur in small numbers along the course of the inflow through the Eastern Channel. In May they are generally distributed in the eastern part of the Gulf which is most accessible to inflowing water and are scarce in the western region in which the larger specimens of the *A* population are dominant.

These observations justify the belief that the *A* population represents the invasion of the Gulf from the eastward by a homogeneous population which grows as it drifts westward.

Reproduction in the Gulf

The appearance of the *B* population in the eastern sectors, unaccompanied by members of the *A* population and the extension of the smaller group westward during May makes it clear that the new population of small individuals which appears in the spring is due chiefly to a second invasion of *Limacina* into the Gulf from waters offshore and to the eastward. Whether the small individuals which occur mixed with the *A* population in several sectors are the offspring of the *A* population is difficult to decide. Dr. Hsiao finds the *A* population to contain sexually active individuals during the spring, so that offspring from this population are to be expected. On the other hand, the relative scarcity in May of the *B* population in the Massachusetts sector where the *A* population was then most concentrated, argues against the possibility that the *B* population in neighboring sectors is in any important degree the offspring of the *A* population. It is noteworthy that when the *A* population first appeared in December it was dominated by individuals of

small size and no individuals of large size occurred among them. This was also true in many hauls in which the *B* population appeared in April and May. This indicates that the parental generation does not survive after breeding long enough to be caught along with offspring which have grown to diameters of 0.3 mm. or greater. This consideration also supports the view that the hauls in which large and small individuals occur together represent the mingling of populations of different origin rather than the simultaneous presence of a parental and filial generation.

Growth

Since the foregoing analysis of the collections appears to warrant the conclusion that the *A* and *B* populations are homogeneous entities,

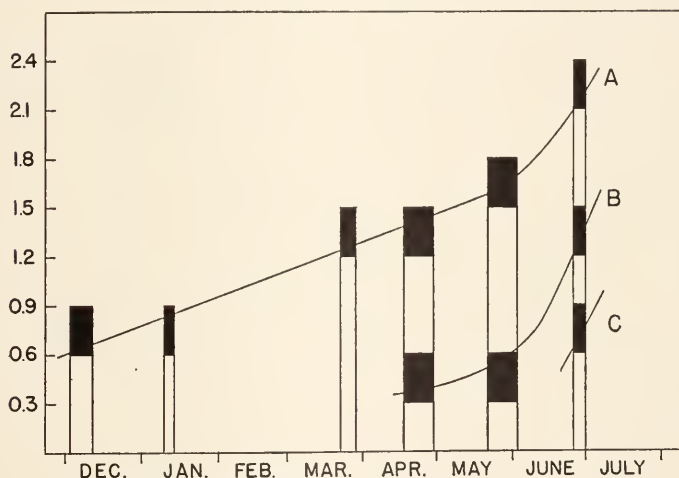


FIG. 9. Diagram indicating the increase in diameter of the modal class of *Limacina* of populations *A*, *B* and *C* during the winter of 1933-34. Ordinates, size groups divided in intervals of 0.3 mm. diameter. Abscissae, time during which collections were made. The black areas represent the modal class size of each population.

we may examine the growth rate of *Limacina* by comparing the size of the modal classes of these populations at different times throughout the year. In Fig. 9 the size of the modal class is plotted against the time the collection was made. Roughly speaking, the diameter of the *A* population doubles in about five months during the winter. At this time the temperature of the water column as a whole is below 7° C. The *B* population observed in May and June grows very much more rapidly as might be expected from the higher temperature of the surface waters at that time.

Numerical Abundance and Mortality

The *A* population is sufficiently established as a distinct and homogeneous entity and was under observation for a sufficiently long period to warrant some statistical examination of its numerical abundance. It must be realized that the number of hauls made in each cruise is insufficient and their distribution too irregular to allow great confidence in the outcome of the analysis of a matter of such complexity, but data of this sort are so rare and so difficult to obtain that one is warranted in the attempt to get the utmost out of it.

TABLE I

Statistics of the size of catches of Limacina retroversa made during the cruises of 1933-1934.

Cruises	No. of Hauls	Total Catch	Mean Catch per Haul	Per Cent of Hauls with Limacina Present	Per Cent of Hauls with Catch Greater than Mean
Sept. 2-14, '33.....	34	104	3	44	21
Dec. 2-11, '33.....	22	3120	142	23	18
Jan. 8-13, '34.....	12	2685	224	66	33
Mar. 21, 29, '34.....	18	2535	141	100	55
April 17-23, '34*—Small.....	24	141	6	71	33
Large.....	24	2575	107	84	24
Total.....	24	2716	113	100	23
May 21-June 3, '34—Small.....	44	4068	92	98	25
Large.....	44	1849	42	91	25
Total.....	44	5917	134	100	23
June 25-July 1, '34.....	7	220	31	100	57
Sept. 17-27, '34.....	26	507	20	92	20

* Two hauls made in the Eastern Channel on May 6, 1934 are included in the data for the April cruise.

We may consider that all the individuals taken in December, January and March belong to the *A* population. In order to eliminate as far as possible the *B* population individuals smaller than 0.9 mm. are eliminated from the data for April and smaller than 1.2 mm. from that for May. The *A* population cannot be identified with assurance in the June collections.

Increase in numbers can be due only to the invasion of the Gulf by new immigrants from offshore since the elimination of the *B* population precludes reproduction as a means of increase. Decreases in numbers may be due to water movements, carrying a part of the population out of the area under observation, or to mortality. Since the population first appears densely distributed at one side of the Gulf, some redistribu-

tion of density between different points of observation is to be expected as the result of lateral mixing of the water. This may be expected to result in an increasing uniformity of distribution.

Table I contains some statistics concerning the size of the catches made during the several cruises. An indication of the numerical abundance of *Limacina* in the Gulf as a whole at each period is given by the numbers in column 4 showing the average number taken per haul. Between December and January the numbers increase, after January there is a progressive decline in abundance. An examination of the distribution of *Limacina* from month to month (Fig. 1) warrants the conclusion that the invasion of the *A* population into the Gulf continues through December and January since large catches are made at these times in the region of inflow. The percentage of stations at which *Limacina* was taken (column 5) increases three-fold during this interval. The invasion appears to terminate in January, since following that period no considerable numbers of individuals referable to the *A* population were taken in the region of inflow into the Gulf. Invasion may consequently be excluded as a significant factor affecting the numbers of the population after January.

The numbers of the population decline steadily from January through May, the mean catch of large individuals referable to population *A* decreasing to less than 20 per cent of the original in four months. Significant losses are attributable to the drift of water out of the Gulf. The continuous occurrence of catches containing *Limacina* in the Georges sector, which lies in the path of outflow through the Eastern Channel, and the concentration of the *A* population in the western and southern quarters of the Gulf in April and May, where it is subject to drift outward by way of the South Channel, favor such losses.

It is probable that large losses in numbers also occur as the result of mortality. Measurements of the total volume of zoöplankton caught throughout the Gulf indicate a general mortality of about 60 per cent during the winter period.

The data in Table I indicate that the percentage of stations at which *Limacina* occurred is not less in April and May than in January. If the circulation into and out of the Gulf were rapid enough to account for the greater part of the loss in numbers, a greater decrease in the area of distribution would be expected. It may be argued that since the area of distribution does not decrease while the numbers caught per station decline five-fold, the decline is due to the death of a large part of the population. This argument, however, must be accepted with great reserve, since the lateral mixing of water may cause small numbers of

animals to be carried into regions in which they did not previously occur, and will tend to enlarge the area of occurrence.

Another approach to the question of mortality may be made through an examination of the distribution of size in the catches as a whole throughout the year. In Fig. 6 the size distribution of the *total* catch is represented. This frequency polygon was prepared by averaging the percentages of the population represented by each size group during each period of collection, a procedure which corrects for the variation in "fishing effort" during different cruises, but does not correct for the irregularity of spacing of the cruises in time. The figure shows that the size most frequently caught is 0.6–0.9 mm. Smaller sizes are less frequent in part because both the *A* and *B* populations are carried into the Gulf half-grown, in part because they are more readily overlooked in sorting the catch and in the case of the smallest class, because many may pass through the mesh of the net. The larger size classes diminish progressively in their relative abundance, as would be expected if the population is subject to continuous mortality. The mode of the *A* population is 0.6–0.9 mm. in January, 1.5–1.8 mm. in May. Specimens of the latter size occur about one-third as abundantly as the former in the catches as a whole. This suggests a mortality of about 66 per cent in the time taken to grow from the former to the latter size, during four winter months. Such statistics, however, are not very convincing since the smaller classes are recruited by invasion and the chance of loss by drifting out of the Gulf increases with time, as does the size of the individuals of the population.

Lateral Mixing

The horizontal mixing of water masses, which has recently attracted the attention of hydrographers, may be expected to have consequences of importance in determining the distribution of organisms. If attention is limited to the consequences of steady residual currents, one can only conclude that the pelagic inhabitants of a region such as the Gulf of Maine which is the seat of a cyclonic eddy must be constantly washed away by the flow of water through it. The rise and decline of the *Limacina* population which we have observed supports this conclusion. The existence of lateral mixing, if of considerable extent, should tend to diminish the tendency of residual currents to transport all of the pelagic population out of such a region. By the transport of a part of the population backstream, or around and across the vortex of an eddy, an opportunity would be afforded for the endemic occurrence of the population in spite of the continuous drainage occasioned by the residual currents.

The effect of lateral mixing must tend continuously to make the distribution of the population more homogeneous. After the introduction of a limited water mass containing a rich population into a large unpopulated region, if lateral mixing alone were in operation one would expect the limits of the populated waters to become less distinct and the distribution to become more general. If samples were taken at random at any time during the mixing process, at first a limited number of samples would contain numbers far in excess of the mean value for the collections as a whole, and the remainder would contain small numbers or none at all. As mixing proceeds, the poorly populated waters would become enriched at the expense of the densely populated regions. In both regions the density of population would change in the direction of the mean population for the entire region and eventually the entire distribution would assume a random character in which the modal population density would correspond with the mean. At this point half the samples would contain more than the mean number, and an equal number less than this value. Subsequent mixing would tend only to diminish the variation of the numbers secured in the different samples.

The catches of *Limacina* have been examined for such evidences of lateral mixing. Examination of the distribution of the population shown in Fig. 1 indicates that the populated region was sharply delimited in December—no *Limacina* occurring in regions immediately in advance of the populated waters. In January moderate catches were made in regions well in advance of the main population, suggesting a gradual disintegration of the boundary zone by lateral mixing. In March the area of intense population covers more than half the Gulf and at the same time the numbers present at each station have diminished and become more uniform as required if lateral mixing is in effect. The same tendencies continue in April and May and are particularly evident when the distribution of small specimens (population *B*) is compared. While previous to March less than half the hauls have contained more than the mean catch for the Gulf as a whole, by March at least half the hauls are of this size—as required by random distribution. One feature of the distribution of large specimens in April and May is of particular interest. In the east central part of the Gulf, on the line between Cashes Ledge and Yarmouth, Nova Scotia, the catches of large specimens are on the whole greater in May than in April in spite of the fact that some mortality is doubtless occurring during this period and the movement is contrary to the expected drift of the water.

As a further test to see if the distribution of population is becoming more homogeneous, the catches have been grouped into classes according

to the number taken per haul and the number of catches in each class has been expressed as a percentage of the total number of catches made during the cruise in question. The result is presented in Fig. 10. The left-hand diagram shows the result when the size of the catch is noted

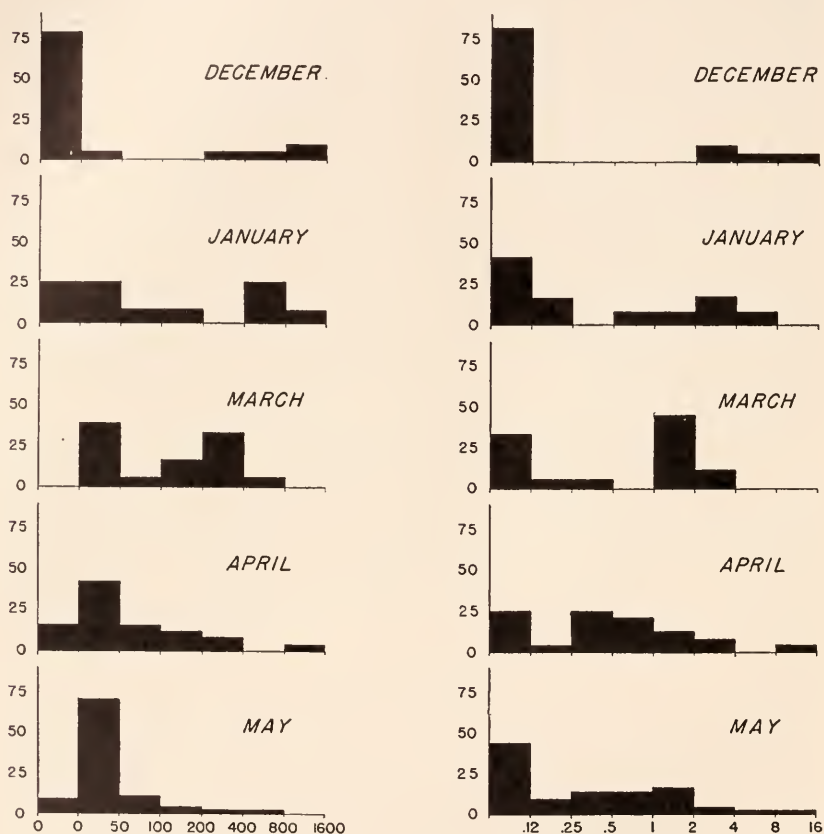


FIG. 10. Frequency polygons showing the distribution of catches of different numerical abundance among the total number of hauls made during each cruise. Ordinates, percentage of entire number of hauls yielding catches of each size. Abscissae, left-hand diagrams, size groups divided in intervals according to number caught in each haul; right-hand diagram, size groups expressed as multiples or fractions of the mean catch per haul during each period.

numerically (and assigned to classes which for convenience increase in geometrical progression). The right-hand diagram represents the same data arranged in size classes which increase as multiples, or decrease as fractions, of the mean population of the region as a whole at the period of collection. The latter procedure eliminates the effect of losses

through mortality. Both diagrams indicate the complete heterogeneity of the samples in December and January and the progressive approach of both the smaller and larger catches toward the mean value which reaches its culmination in April and May.

Actually the situation observed is complicated by the drift of the population across the region and by the invasion of the eastern part of the Gulf by water poor in *Limacina* of population *A* in March and April. This inflow tends to decrease the homogeneity of distribution and accounts for the increase in the proportion of catches in April and May which contained few or no *Limacina*. It also explains why the percentage of hauls in which the catch was greater than the mean diminished in the months subsequent to March. These tendencies during April and May at least make it clear that lateral mixing is not sufficiently great to obliterate the effect of the residual current in depleting the *Limacina* population of the eastern portion of the Gulf.

Because of the impossibility of separating the effects of mortality and of losses and gains through the residual movement of the water into and out of the Gulf from the apparent effects of lateral mixing, any attempt to evaluate the extent of the latter has been abandoned. Because of the great influence which such mixing would have in enabling a population to maintain itself in a region from which the residual current would constantly tend to remove it, it seems desirable, however, to emphasize the possibility of such mixing and to indicate the way in which it appears to manifest itself.

Discussion

The evidence seems definite that the population of *Limacina* which occurred in the Gulf of Maine in 1933-34 was not endemic, as Bigelow thought, but owed its origin to two separate invasions of young individuals from offshore. Unlike the less successful immigrants of arctic or tropical origin, *Limacina retroversa* thrives well enough to grow to sexual maturity and perhaps to reproduce within the Gulf and to be carried in considerable numbers to all its parts. For this reason Bigelow obtained evidence which led him to conclude the species was endemic. By following the history of these populations closely, however, it is shown that neither invasion was able to maintain its numbers in the Gulf—either because reproduction was not successful or was inadequate to balance the depletion due to mortality and the drift out of the Gulf.

The two invasions consisted of dense swarms of small individuals of very uniform size, sharply separated by a period when practically no *Limacina* occurred in the region of inflow into the Gulf. The discon-

tinuous character of these invasions no doubt accounts for the irregularity in occurrence of *Limacina* in the Gulf noted by Bigelow. Why the invasion occurs in such definite periodic swarms can be answered only by studies carried out in the waters to the eastward from which these invasions undoubtedly come.

It is an interesting question why the invading swarms are composed of individuals of such uniform size. In describing the population found in the Gulf in September, 1934, it was pointed out that animals of all sizes were mixed together and that successive broods can no longer be distinguished. Dr. Hsiao's studies indicate that the mature *Limacina* continue to produce eggs during a considerable period of growth. Yet we found evidence to suggest that the parental generation does not survive after breeding long enough to be caught along with its offspring at the time when these enter the Gulf. Dr. Hsiao has measured numerous catches of *Limacina* taken over the continental shelf between Cape Cod and Cape Hatteras. Usually these catches are composed of individuals of uniform size, just as were those which invaded the Gulf. This riddle also can be answered only by closely timed observations in the offshore areas of production.

The most conspicuous result of this study is the demonstration of the degree to which the occurrence of *Limacina* in the Gulf of Maine depends upon the circulation of its waters. Damas in 1905 raised the question: How does the plankton of a given region maintain its character in the face of the continual circulation of the currents and how does a given species persist so as to possess a special geographic distribution? He concluded that there must exist a special zone or center of production in which adults abound and reproduce successfully and that to this region circulatory currents serve to bring back periodically a proportion of the individuals which become entrained and dispersed by the continual movements of the water. The circulatory system of the Norwegian Sea subsequently described by Helland-Hansen and Nansen (1909) appeared to supply just such a mechanism as was required to account for Damas' observation of the production centers of copepods in that region. Sømme (1933, 1934) has shown how such centers of production are established for *Calanus finmarchicus* and *hyperboreus* in the deep waters of the Norwegian fjords in winter, and has elucidated the mechanism which results in the release of the organisms from these regions at the time of reproduction. The observations on *Limacina* have not revealed the presence of a center of production in the Gulf of Maine. They point to the existence of such regions offshore to the eastward and are of interest rather in telling something of the fate of these animals, entrained in the movement of water, which are carried away

never to return, yet for a while to occupy an important rôle in the ecology of other regions. Behind the geographical distribution of each species of plankton there must be a complex balance of biological and physical factors. Of the latter, flow of water appears to be paramount; its consequences too frequently neglected.

Summary

A population of small specimens of the pteropod, *Limacina retro-versa*, appeared in the eastern part of the Gulf of Maine in December, 1933. From collections made during the following 9 months information was obtained showing that the population was a homogeneous one, that its members grew to maximum size in 5 months, declining in numbers as they did so.

A second population of small individuals appeared in the Gulf in late spring, originating chiefly from offshore, but possibly in part being offspring of the original population. These were unsuccessful in maintaining their numbers throughout the summer.

In addition to the information on the life history of *Limacina*, the data indicate the rate of drift of the water in its circuit of the Gulf. It supplies also suggestive information on the dispersal of organisms through the lateral mixing of water. It emphasizes the dependence of pelagic organisms upon the current systems of the ocean and the difficulty involved in maintaining a permanent population in any one locality.

REFERENCES

- BIGELOW, H. B., 1926. Plankton of the offshore waters of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40** (1924), Part II: 1-509.
- BIGELOW, H. B., 1927. Physical oceanography of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40** (1924), Part II: 511-1027.
- DAMAS, D., 1905. Notes biologiques sur les copepods de la Mer Norwegienne. *Publ. de Circonstance*, No. 22, Copenhagen.
- HELLAND-HANSEN, B., AND F. NANSEN, 1909. The Norwegian Sea. Report of the Norwegian Fishery and Marine Investigations. II. Part I.
- HUNTSMAN, A. G., 1924. Oceanography. *Handbook of the British Association for the Advancement of Science*, Toronto, pp. 274-290.
- KÜNNE, CL., 1933. Weitere Untersuchungen zum vergleich der Fangfähigkeit verschiedener modelle von vertikal Fischen den Plankton-netzen. *Rapp. et Procès-verb. des Reunions. Conseil perm. internat. pour l'explor. de la mer*, **83**: 19 pp.
- PAULSEN, O., 1910. Pteropoda. *Bull. Trimestriel, Conseil perm. internat. pour l'explor. de la mer*, Part I, pp. 52-59.
- SØMMER, I. D., 1933. A possible relation between the production of animal plankton and the current-system of the sea. *Am. Nat.*, **67**: 30.
- SØMMER, J. D., 1934. Animal plankton of the Norwegian coast waters and the open sea. I. Production of *Calanus finmarchicus* (Gunner) and *Calanus hyperboreus* (Kroyer) in the Lofoten area. *Report on Norwegian Fishery and Marine Investigations*, IV, No. 9, pp. 3-163.

RESPONSES OF THE SWIMBLADDER OF THE GUPPY, *LEBISTES RETICULATUS*, TO SUDDEN PRESSURE DECREASES

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Most fishes maintain a density equal to that of the surrounding water. This is done through a very accurate control of the volume of their swimbladders by appropriate gaseous exchange between the swimbladder and the blood. As the fish passes into deeper water the increased hydrostatic pressure compresses the bladder gases to the point that the fishes are no longer buoyed up in the water. Consequently, in order for the fishes to adapt themselves to the new pressure, they must put more gases into the bladder. Conversely, as the fishes rise in water the decreasing hydrostatic pressure renders the gases in the bladder too buoyant and provision is made for the release of the proper amount. Many experiments have been performed in an attempt to determine the mechanism of control of the swimbladder. The great majority of these experiments have involved analysis of gases found in the swimbladder in different states of adaptation and under controlled experimental conditions. Although much valuable information has been obtained, our picture of the mechanism is by no means complete.

Von Ledeber (1937) summarizes our present knowledge in this field and reviews briefly all the current theories to explain the secretion of gases into the swimbladder against the diffusion gradient and their removal. The variety of explanations indicates the need of more research before a definitive theory can be devised.

The experiments to be described in this report were carried out upon the guppy, *Lebistes reticulatus*, a physoclistous fish. They demonstrate the responses of the swimbladder to decreased hydrostatic pressure and to situations in which the normal diffusion gradient, favoring passage of gases out of the bladder, is experimentally reversed and varied in steepness. Some interesting information has been obtained upon the mechanism of gaseous exchange between the environment and swimbladder.

The apparatus consisted of a two and a half gallon carboy. Through the stopper was projected a small bore glass tube which passed into a flask serving as an air cushion. To this flask was attached a mercury manometer and tubes which led to a vacuum pump on the one hand and

a compressor on the other. Both of the latter tubes were equipped with stopcocks. The whole system was arranged so that the pressure within the system could be rapidly changed but always kept under complete control. The experimental animal was placed in the carboy completely filled with water which rose three or four inches into the small bore glass tube of the stopper. In a few experiments where the gases present in the water were to be equilibrated to each new pressure by shaking, a liter bottle half full of water was substituted for the carboy.

With this equipment it was possible either to alter the pressure upon the fish without appreciably altering the gaseous concentration of the medium,¹ or to subject the animal to atmospheric pressure in water which had been equilibrated with air under heightened or lowered pressure.

With this same apparatus it was a simple matter to measure any changes in the total amount of gases in the bladder by the application of Boyle's law. The method was merely a refinement of the technique used by Evans and Damant (1928). From time to time the exact pressure to which the guppy was adapted was measured. As the amount of gases in the bladder increased, proportionately more pressure had to be applied in order to return the fish to the same density as the water, and conversely, as the fish permitted gases to escape from the bladder, proportionate decrease in pressure was necessary to adjust artificially the fish density so that the fish would tend neither to rise nor sink. The validity of this method depends upon the assumption that the pressure of the bladder gases results solely from the external environment about the fish. Evans and Damant found this to be the case with the physoclistous fish they examined, the bladder gas not being held under pressure by any structure of the fish body.

Some initial experiments were performed with completely normal guppies, but it was found difficult to make accurate readings of the exact pressure to which the fish was adapted at any given moment. This difficulty was a result of the fish's being able to maintain itself quite stationary in the water through an appreciable range of pressures by very slight activity of the pectoral fins. Consequently, all experiments reported here were conducted with fishes whose pectoral fins were amputated. This last procedure permitted equilibrium readings of considerable accuracy in a few seconds of time. There has been no reason to suspect that this fin removal affects the swimbladder response in any manner.

Such a method as the one used here may be applied to any physoclistous fishes since in these fishes the change in gas quantity takes place

¹ When not otherwise specified in the following experiments, the gaseous content of the water was that resulting from equilibrium with air at room temperature and the atmospheric pressure (about 750 mm. Hg).

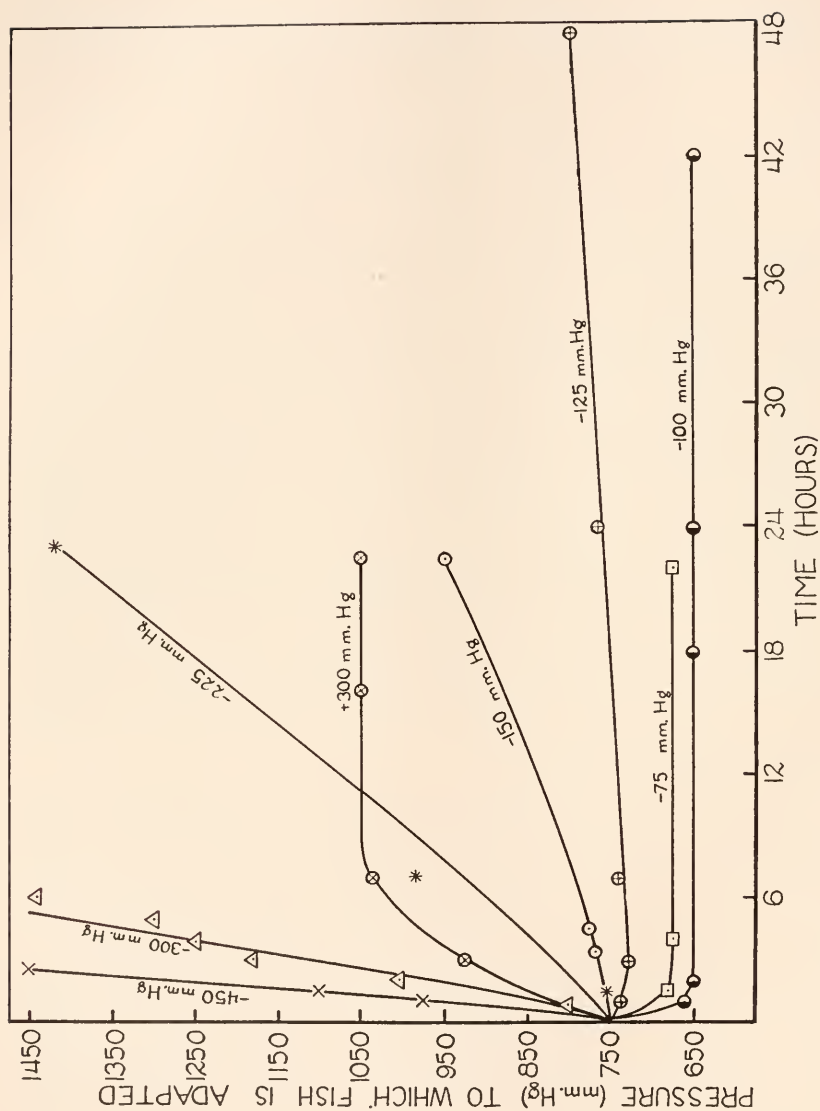


FIG. 1. Diagram showing the responses of the swimbladder to decreases in pressure of 75, 100, 125, 150, 225, 300, and 450 mm. Hg, and an increase of 300 mm. Hg.

only very slowly by secretion or diffusion of gases and hence the few seconds of sudden change of pressure for the purpose of determining the direction and extent of gas change cannot result in an abrupt change in gas content. Physostomous fishes such as the goldfish, on the other hand, almost instantly discharge gas bubbles by way of the mouth when the pressure is suddenly decreased.

EXPERIMENTAL

The first experimental series was designed to test the effect of changes in pressure upon the fish swimbladder response. Water was equilibrated with air at room temperature and at atmospheric pressure and placed in the carboy. A female guppy was permitted to adjust its density to that of the water in the new situation: a pressure of one atmosphere plus eighteen inches of water. This situation served as a starting point for each experiment. In as many separate experiments the pressure on the fishes was decreased by 75, 100, 125, 150, 225, 300, and 450 mm. Hg and increased by 300 mm. Hg. The responses of the fish to these changes are indicated in Fig. 1.

The fishes responded to increased pressure by decreasing their density gradually over the course of six or eight hours to the point of equaling that of the water. This density was maintained as long as the pressure was held constant. When the pressure was decreased by 75 or 100 mm. Hg, the fish increased its density to a value equal to the surrounding water.

However, as the pressure was decreased more, by 125, 150, 225, 300, and 450 mm. Hg, the fish no longer adaptively adjusted its density. Instead, its density decreased at a rate that was obviously a function of the amount of decrease in pressure. These results were confirmed by a second experiment in which only male guppies were used.

With pressure decreases of more than 100 mm. Hg the density of the fish continued to decrease for several days at a nearly constant rate, or until the swimbladder became so large that the wall of the visceral cavity was distended in a balloon-like fashion. When the pressure was decreased by 300 or more mm. Hg such a state was reached in less than twenty-four hours.

There thus appeared to be some level of pressure below which the fish was unable to adapt itself, and on the contrary, became more and more maladjusted. This pressure level appeared to be quite constant whether it was reached in one sudden pressure change, in two equal changes over several hours, or in three changes over a period of four days. Figures 2*A* and 2*B* illustrate results of this nature that were

obtained. Furthermore, the rate of decrease in fish density in response to a decreased pressure appeared to be independent of the rate at which the value was approached.

INTERPRETATION OF THE EXPERIMENTAL RESULTS

The cause of the abrupt reversal of fish response from density increase to decrease as the pressure was decreased by values greater than

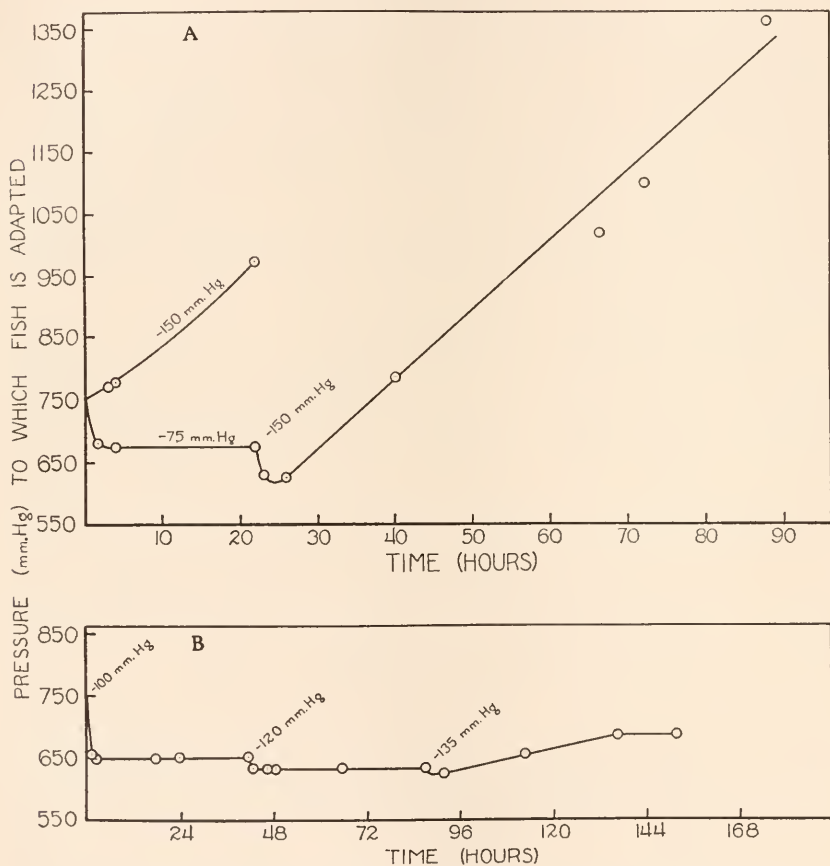


FIG. 2. (A) Graph showing the swimbladder responses to reduction in pressure when the reduction is carried out in a single step, in two steps over a period of about 20 hours, and (B) three steps over a period of about four days.

about 120 mm. Hg was the first question to be answered. A guppy adapted to a pressure increase of 300 mm. Hg, when suddenly returned to the original pressure, responded by increasing its density to a value

again equal to that of water. On the contrary, a guppy directly subjected to a similar percentage decrease in pressure responded in an opposite fashion by decreasing its density. Therefore, a physically induced size increase of the swimbladder and any activity or response of the fish directly correlated with such increase would not totally explain the situation. This eliminated any reflex associated with the eyes, fins, etc., as was indicated by Meesters and Nagel (1934) to be true over the normal pressure range.

A brief additional experiment was performed which immediately eliminated any induced change in amount of gases dissolved in the water as stimulating the bloating phenomenon. A guppy in an unstoppered flask of water was placed in a vacuum desiccator. The pressure on the fish was then decreased by 300 mm. Hg. The fish commenced to bloat. The water now had a dissolved air content found in conjunction with a decreased pressure which stimulated the bloating of the fish. The flask was quickly removed from the desiccator and paraffin oil poured upon the water surface to prevent any re-diffusion of gases into the water at the restored pressure. If decreased concentration of any gases in the water was responsible for the bloating phenomenon, such bloating would be expected to continue. The fish in this situation increased its density to a value equal again to that of water.

It was highly improbable that the fish was responding to pressure through the activity of any sense organ. The evidence indicated that if a postulated hydrostatic pressure sense organ was actually operating it displayed no signs of adaptation or fatigue (see especially Fig. 2*B*). A physical explanation thus appeared more plausible.

Two further and decisive experiments were performed which demonstrated the character of this pressure effect. A guppy was placed in a bottle only partially filled with water. The water was thoroughly shaken with air at each reduced pressure. The fish adjusted its density to agree with that of the water for all the pressure decreases used. In one experiment the pressure was decreased by 300 mm. Hg. Again, when water was thoroughly shaken with air under a pressure increase of 500 mm. Hg, the guppy bloated when placed in this after the pressure had been restored to the original value. The rate of bloating was of the same order of magnitude as seen in response to a pressure decrease of 300 mm. Hg.

A partial interpretation of the experimental results thus seems evident. Except for the pressure exerted by the short column of water over the fish in the experiments, the gas in the bladder is of the same pressure as the atmospheric air at the water surface. Under reduced pressure, tension of gases in the water favors diffusion of gases into the

bladder and atmosphere alike. The greater the decrease in pressure, the more rapid the passage of gases into the bladder.

Not up to this point explained, however, is the initial lower rate of bloating, sometimes even temporary gas output, which occurred when guppies were subjected to decreases in pressure sufficiently large to produce the bloating phenomenon. The temporary output is seen in Fig. 1 (reduction in pressure by 125 mm. Hg) and in Figs. 2*A* and 2*B* at decreases in pressure by the amount 150 (when this was carried out in two steps) and 135 mm. Hg. This temporary initial activity, an apparent attempt of the fish at adaptation, commences strongly and then gradually over the course of two to five hours becomes weaker until it is completely masked by the inward passage of gases. Either an outwardly secreting mechanism which could secrete quite efficiently up to a point of fatigue, or simple diffusion alone, might account for the response. The former explanation can neglect the partial pressures of the various gases within the swimbladder. The latter explanation is based upon the assumption that at the beginning of the experiment the bladder possesses a higher percentage of CO_2 than does air.

Recent work of Meesters and Nagel (1934) has shown that a fish would, under the appropriate stimulation, decrease the amount of bladder gas most rapidly when the initial percentage of CO_2 in the bladder was high. Thus, fish were apparently able to eliminate this gas much more rapidly than either O_2 or N_2 . The work of Jacobs (1932), confirmed by Meesters and Nagel (1934), has demonstrated that a physio-

FIG. 3. Responses of the swimbladder to sudden decreases in pressure following immediately upon different experimental conditions of secretion and diffusion of gases to and from the bladder.

A. Two experiments in which guppies are subjected to a pressure decrease of 300 mm. Hg in water equilibrated with air at the reduced pressure, then returned to atmospheric pressure in water equilibrated with the restored pressure. At the moment of adaptation to the restored pressure the fishes are subjected to a sudden pressure decrease of 300 mm. Hg.

B. A single experiment in which a guppy was subjected to pressure increase of 260 mm. Hg and at the moment of adaptation subjected to a decrease to a point 300 mm. Hg below atmospheric pressure.

C. Three experiments in which guppies long (20 hours to several days) adapted to a given pressure are subjected to a sudden pressure decrease to a point 300 mm. Hg below atmospheric pressure.

D. Two experiments in which guppies are stimulated to decrease the gas content of their bladders and then suddenly subjected to a pressure decrease to a point 300 mm. below atmospheric pressure.

E. The two upper graphs indicate the response of guppies, bloating as a result of a 300 mm. Hg decrease in pressure, when they are suddenly returned to atmospheric pressure. The lower graph shows the normal response of the fish upon increase in pressure of 300 mm. Hg and then, after some hours, return to atmospheric pressure.

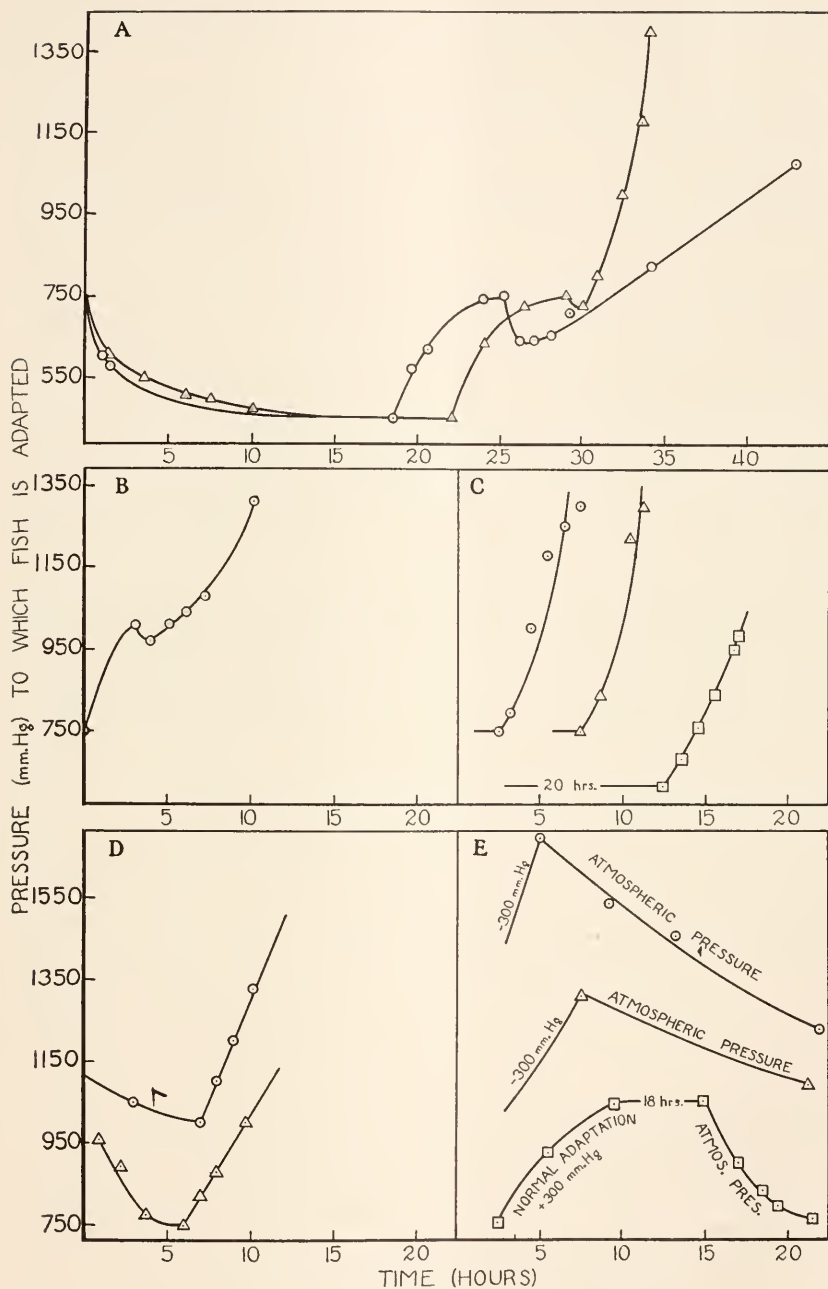


FIG. 3.

clitinous fish, the perch, secretes into its swimbladder gas which consists of about eighty per cent CO_2 .

These results may be applied to a physical interpretation of the observations that have been made upon the guppy. One would merely need to assume that the fish usually possesses in its bladder a partial pressure of CO_2 several times that in air at the water surface. The pressure decreases of the experiments (never more than about sixty per cent) would still favor the passage of CO_2 out of the bladder while one or both of the remaining two gases would diffuse inward. The magnitude of the partial pressure of CO_2 inside and the pressure decrease would determine the rate of outward diffusion of this gas and this rate might even be great enough to make the volume of the bladder decrease. Meanwhile, the response of the secretory mechanism to the pressure decrease would be one of minimal activity. As the CO_2 became depleted the rate of its outward diffusion would decrease correspondingly. The inward diffusion of the gases other than CO_2 would also tend to decrease the partial pressure of CO_2 in the bladder. Eventually, the partial pressures of all three gases would attain the same ratio as that of atmospheric air and then all gases would diffuse inward at rates to maintain this ratio.

Indirectly, some evidence has been obtained to support the hypothesis just described. We would expect that increasing the proportion of CO_2 in the bladder would permit a stronger opposition to the decrease in pressure, and a sufficient reduction in the bladder CO_2 would eliminate it altogether. The following are experiments which give such support.

Two male guppies were subjected to a pressure decrease of 300 mm. Hg in water brought into gaseous equilibrium at the reduced pressure. First there was a rapid increase in fish density and then after an hour or two a considerably slower one. These two rates were interpreted on the basis of the work of Meesters and Nagel (1934) to indicate an initial rapid escape of CO_2 and then the slower continued escape of other gases. The bladder was thus assumed to become relatively free of CO_2 and also to contain a smaller quantity of the other gases than originally. The fish was then restored to the original pressure in water equilibrated with air at the restored pressure. The gas secreted into the bladder was assumed on the basis of Jacobs' (1932) results to be richer in CO_2 than that which was withdrawn. When the proper volume of gas for the new pressure was reached and before the amount of CO_2 could decrease to equilibrium with a slower secretory rate, this fish was subjected to the sudden pressure decrease of 300 mm. Hg. This fish was able to oppose much more strongly the bloating associated with this decreased pressure than was the fish of the earlier experiments. Compare the results of experiments of this nature (Fig. 3A) with the results ob-

tained when guppies had neither appreciable gain nor loss in bladder gases over some days (Fig. 3C) but subjected to the same pressure decrease.

The same general results were obtained in a slightly different manner. A guppy was stimulated to decrease its density by subjecting it to an increase in pressure of 260 mm. Hg. At the moment of adaptation to the new pressure the fish was subjected to a pressure reduction of 560 mm. Hg (Fig. 3B). This was the equivalent of the usual reduction in pressure of 300 mm. Hg.

The reverse type of experiment was next performed. Guppies, for some hours adapted to increased pressures, were stimulated to let gases escape from their bladders. On the assumption that CO_2 is the gas to pass out most rapidly these fishes should, after considerable increase in density, have no larger percentage of CO_2 than is present in atmospheric air. And as expected the fish were at this moment unable to oppose in the slightest the bloating effect correlated with subjection to a sudden 300 mm. Hg pressure decrease. Figure 3D illustrates results of this nature.

A final test for the hypothesis was made to ascertain that the gas present in the fish bloating rapidly under decreased pressure did not contain as large a percentage of CO_2 as did the normally secreted gas. A guppy that had reached the maximum rate of bloating under the stimulus of the reduced pressure was suddenly restored to the original pressure. Under ordinary circumstances the initial response to such a decrease in pressure is a relatively rapid fish density increase which gradually or suddenly slows down. In the instance of this fish the initial response showed none of the usual initial rapidity and the whole process of return to the normal amount of gas for the pressure was relatively slow, as one would expect if the rate were determined by gases which were slow to diffuse (Fig. 3E). The normal rate of gas escape following secretion of gases into the bladder eighteen hours previously is also shown in the same figure for comparison.

SUMMARY

1. A sudden and maintained pressure decrease from a value of one atmosphere to points between 625 and 300 mm. Hg results in an increase in gas content of guppy swimbladders and the rate of increase is an inverse function of the pressure.
2. This increase in gas content has been shown to be the result of lowering the swimbladder gas pressure, the gas tension of the body fluids then favoring passage of gases into the bladder.

3. Guppies are able to oppose more or less successfully the inward diffusion of gases at first, but gradually in the course of two to five hours such opposition ceases.

4. The transitory attempt on the part of the fish at density adjustment in response to the pressure decreases is explainable in the presence of an initially high partial pressure of CO_2 in the swimbladder.

5. The guppy appears to be unable to remove gases from the bladder when the diffusion gradient does not favor such passage.

LITERATURE CITED

- EVANS, H. M., AND G. C. C. DAMANT, 1928. Observations on the physiology of the swimbladder in Cyprinoid fishes. *Brit. Jour. Exper. Biol.*, **6**: 42-45.
- JACOBS, WERNER, 1932. Untersuchungen zur Physiologie der Schwimmblase der Fische. II. Die Volumregulation in der Schwimmblase des Flussbarsches. *Zeitschr. vergl. Physiol.*, **18**: 125-156.
- MEESTERS, A., AND F. G. P. NAGEL, 1934. Über Sekretion und Resorption in der Schwimmblase des Flussbarsches. *Zeitschr. vergl. Physiol.*, **21**: 646-657.
- VON LEDEBUR, J., 1937. Über die Sekretion und Resorption von Gasen in der Fischeschwimmblase. *Biol. Rev.*, **12**: 217-244.

THE INTERNAL ANATOMY OF TWO PHALLOSTETHID FISHES

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INTRODUCTION

The Phallostethidae are highly specialized little fishes found in fresh and brackish water in certain limited regions of the Malay peninsula and the Philippines. They are of general interest in that they appear to provide a unique example of change of structure and function in a typical teleost organ, the pelvic fins of the male. The females are also modified, but to a lesser degree. The fishes derive their name from a large, muscular copulatory organ, the "priapium," suspended below the head in the male and which contains, in addition to the ductus deferens, the terminal apertures of the digestive and excretory systems (Figs. 1 and 2). In the female, the organ outlets are also anterior but, since there is no copulatory organ, the apertures are located on the ventral surface of the gular region (Fig. 3).

The phallostethids were first described and named by Regan in 1913 from a single species, but he notes a clear reference to their type by Duncker in 1904. Since Regan's work, twelve species have been discovered (Myers, 1928, 1937; Aurich, 1937). All are small, measuring from 14 to 40 mm. in length, and in 1935, after considerable study, Myers created for the group a new sub-order, Phallostethoidea, in the order Percosces. Myers lists seven genera; Aurich groups them into three families and adds an eighth genus, *Solenophallus*.

Investigations on this group have been largely concerned with attempts to discover its taxonomic relationships, and a search has been made for possible homologies between the bony elements supporting the priapium and the bones of more typical teleosts.

Regan (1916), Bailey (1936) and Aurich (1937) have described the osteology of the priapium in several species of phallostethids. Bailey and Aurich each conclude that the priapial bones represent greatly modified pelvic fin and girdle elements, together with the first pair of ribs and, Bailey adds, possibly certain pterygials or rays of a portion of the pectoral fins. Aurich strengthens the argument that pelvic fin elements

have been transformed into priapial structures by describing the presence of vestigial fin rays in two species of the genus *Solenophallus*, and in *Gulaphallus mirabilis*. In all three species, he finds two or three delicate rays lying free in the skin behind the anus, and a second group of three rays inserted on the posterior extremity of the axial bone, a skeletal support which extends throughout the length of the priapium (see Aurich Fig. 3). In *Solenophallus*, these latter rays become comb-like at the ends. The females of several species possess a pair of papillae on either side of the oviduct opening (Regan, 1913; Herre, 1925, 1926; Villadolid and Manacop, 1934; Bailey, 1936). These may be vestiges of pelvic fins, and it is thought (Bailey) that the minute bony supports found in them in *Phenacostethus* may be remnants of the pelvic girdle. Villadolid and Manacop (1934) describe the development of the copulatory organ in *Gulaphallus*, showing it to arise from paired lobes surrounding the anus, on the ventral side of the throat. One of these lobes soon outgrows the other; the two fuse and gradually produce the elongate, asymmetrical priapium.

Brief descriptions of the soft parts of phallostethids have been published by Regan (1916) for *Phallostethus dunckeri* and *Neostethus lankesteri*, and by Villadolid and Manacop (1934) and Bailey (1936) for *Gulaphallus mirabilis*. There has been no detailed study of the gross and histological structure of the viscera, however, and it is to Dr. Hugh M. Smith that I owe the suggestion that such a study in these greatly modified forms would be of interest. Dr. Smith referred me to Dr. George S. Myers who supplied preserved material of two species, *Phenacostethus smithi*, and *Gulaphallus mirabilis*, from the collection at the U. S. National Museum. I wish also to acknowledge with appreciation the help Dr. Myers has given me in sending me a number of papers on the phallostethids.

MATERIAL AND METHODS

Phenacostethus smithi, described by Myers in 1928 from material collected by H. M. Smith in a fresh water stream in Siam, is the smallest phallostethid known (14–17 mm. total length) and is next to the minute Philippine goby, *Mistichthys luzonensis* (11–16 mm. total) in size. *Gulaphallus mirabilis* was described in 1925 by Herre as one of two species of a new genus which he had found in the fresh water streams of Luzon, the Philippine Islands. Its total length is not over 35 mm.

The specimens at my disposal were fixed in formalin and alcohol and preserved in alcohol. Transparent preparations of the skeleton were made by staining with alizarin and clearing in KOH and glycerine.

These were studied and dissected under a binocular dissecting microscope and other unstained specimens were dissected in alcohol for a study of organ arrangement. Sketches were made with the aid of a camera lucida. Serial sections were cut in transverse, sagittal, and frontal planes, 7–12 μ thick, and stained in iron haematoxylin, alum haematoxylin and triosin, and in Mallory's triple connective tissue stain. In all cases, fixation was poor for an adequate histological study and much more satisfactory results could be obtained with properly fixed material.

SKELETON

The descriptions of almost the entire skeleton of *P. smithi* and of *G. mirabilis* by Bailey (1936) and of the priapial skeleton of the latter species by Aurich (1937) give detailed information to which I can add nothing except to mention that Bailey did not notice the vestigial fin rays which Aurich finds in the priapium of *G. mirabilis*. After very careful examination, I have identified both the loose post-anal group of rays and the group articulated with the posterior end of the axial bone in that species, thus supporting Aurich (see Aurich, 1937, Figs. 3 and 8). In the priapium of *Phenacostethus*, however, I have found no trace of fin rays. This fish is much smaller than *Gulaphallus* and the skeletal elements are correspondingly more delicate, which may in part account for the disappearance of such minute vestiges.

PSEUDOBANCH

The pseudobranch, which Aurich says is lacking in phallostethids examined by him, including *Gulaphallus*, is present in both that species and in *Phenacostethus* in the form of two minute tufts (Myers, 1928) lying in a recess at the anterior dorsal end of the sub-opercular cavity. Microscopic study shows this organ to belong to type II of Granel's (1927) classification of teleost pseudobranchs, in that the epithelium of the branchial chamber surrounds each tuft separately, but does not extend between the secondary lamellae of the tuft. Each primary lamella, or tuft, is furnished with an afferent artery and is supported by a pre-cartilage rod which is extremely delicate in *Phenacostethus*. The secondary lamellae are close together and contain the large acidophile cells typical of the pseudobranch.

VISCERA

The general arrangement of organs in *Phenacostethus* is shown in Figs. 1 and 2. Comparison with Regan's (1916) figures of *Phallo-*

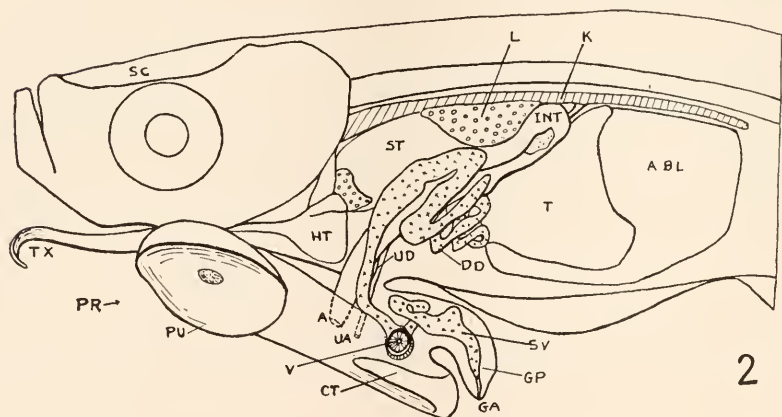
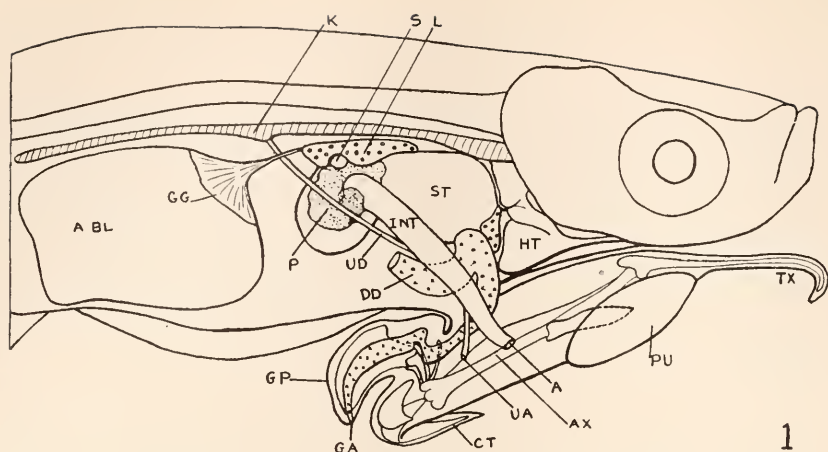


FIG. 1. *Phenacostethus smithi* ♂, internal organs in situ from the proctal side ($\times 16$). Testis and portion of ductus deferens removed. The priapium is the large ventral appendage; several bones in it are outlined but Bailey's figures should be consulted for the complete skeleton.

FIG. 2. *Phenacostethus* ♂, internal organs in situ from the aprocital side.

Abbreviations

<i>A</i> anus	<i>PR</i> priapium
<i>ABL</i> air bladder	<i>PU</i> sucker-like pulvinulus
<i>AX</i> axial bone	<i>S</i> spleen
<i>CT</i> ctenactinium	<i>SC</i> region of sensory canals
<i>DD</i> ductus deferens	<i>ST</i> stomach
<i>GA</i> genital aperture	<i>SV</i> seminal vesicle
<i>GG</i> gas gland	<i>T</i> testis
<i>GP</i> genital papilla	<i>TX</i> toxactinium
<i>HT</i> heart	<i>UA</i> urinary aperture
<i>INT</i> intestine	<i>UD</i> urinary duct
<i>K</i> kidney	<i>V</i> valve in ductus deferens
<i>L</i> liver	

stethus dunckeri and with Villadolid and Manacop's (1934) figures of *Gulaphallus mirabilis* will give evidence of the great general similarity in the visceral anatomy of the group. Digestive and reproductive organs are located almost entirely in the space anterior to the air-bladder and, together with the excretory ducts, these systems terminate in apertures situated on the ventral surface of the throat region. It has already been pointed out (p. 59) that this extremely anterior position is associated with the male copulatory organ or priapium which is suspended from the head. The outlets are asymmetrically placed on the priapium; the anus and urinary duct open on one side, the proctal, while the ductus deferens ends in a genital papilla on the opposite or aproctal surface. As other workers have noted, this asymmetry is variable, that is, in some males the right side is proctal, in others, the left. In the female the apertures are mid-gular although no copulatory organ is present (Fig. 3).

The following more detailed description applies to both *Phenacostethus smithi* and *Gulaphallus mirabilis* unless attention is called to one species only.

ENDODERMAL DERIVATIVES

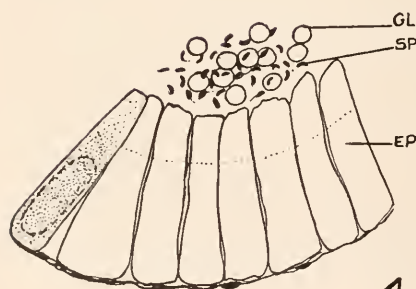
The digestive tract is simple, short, and without pyloric caeca; a typical carnivorous type, as is borne out by Villadolid and Manacop in their analysis of the food of *Gulaphallus*. The intestine makes one complete coil in *Gulaphallus* and a partial coil in *Phenacostethus* (Fig. 1) before turning anteriorly toward the anus. The liver is large and lies close to the stomach and intestine (mainly on the aproctal side in the male); no gall bladder was identified. The pancreas is diffuse tissue, lying in the bend between stomach and intestine, with considerable fat scattered among its cells. Perhaps because of unsatisfactory fixation, no islet tissue is distinguishable. The spleen is partly embedded in the liver posterior to the stomach (Fig. 1). Thymus and thyroid glands are present. The large air bladder fills nearly one half of the body cavity. It lacks a pneumatic duct but is equipped in the anterior dorsal region with a vascular gas gland.

NERVOUS SYSTEM

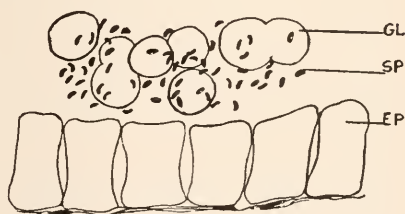
The size of the brain is large in proportion to the skull as in other minute fishes (TeWinkel, 1935). It was impossible because of poor fixation to determine whether the brain and cord are modified in relation to the priapium. Neuromasts are scattered on the surface of the body, particularly on the head region in *Gulaphallus*; a few are present in *Phenacostethus*, but here too, fixation was imperfect. Four large canals,



3



4



5

FIG. 3. *Phenacostethus smithi* ♀, ventral view, anterior end ($\times 16$). A, anus; HT, heart; N, neuromast; O, oviduct aperture; PAP, post anal papilla; UA, urinary aperture.

FIG. 4. Portion of proximal coil of ductus deferens in *P. smithi*. Ep, epithelium, showing in one cell probable position of nucleus; dotted line represents depth of lighter area in cells; GL, globules; SP, sperm head. Drawn with camera lucida under oil immersion lens.

FIG. 5. Portion of distal coil of ductus deferens from same section of *P. smithi* as Fig. 4. Epithelial cells are much lower and globules between sperm heads much larger than in the preceding figure. (Same magnification as above.)

with patches of sensory cells (which are probably lateral line organs) on their ventral inner walls, extend longitudinally on the snout and above the eyes in both species. These occupy considerable space between the skin and skull, and occasionally a homogeneous mass, possibly a cupula (Denny, 1937), is present in contact with the sense organ. The cavity containing the inner ear is proportionally large as in *Mistichthys*.

KIDNEY

The kidney in both species is a median organ which lies dorsal to the air bladder and digestive tract and diverges anteriorly into two sections which extend from below the occipital region of the skull up to the posterior level of the gills (Fig. 1). In *Phenacostethus*, and possibly in *Gulaphallus*, the urinary ducts are paired upon their exit from the kidney a short distance in front of the air bladder. They soon fuse, however, into a single duct which, in both species, passes between the intestine and gonad to open in the male on the priapium, directly behind the anus (Figs. 1, 2), and in the female, posterior to the oviduct in the gular region (Fig. 3).

Serial sections reveal one pair of pronephric glomeruli in both species. These lie dorsal to the heart and are directly attached to the dorsal aorta, which position is characteristic of the teleost pronephros. Poor fixation makes it impossible to tell whether the pronephric tubules are being replaced by lymphoid tissue, or whether they are functional, except the fact that the glomerular capillaries are full of blood cells, which suggests some function. The mesonephric glomeruli are smaller than the pronephric and are most numerous in that portion of the kidney anterior to the air bladder. The number of these glomeruli varies in *Phenacostethus* from 13 to 18, and in *Gulaphallus*, from 33 to 38, in the specimens where counts were made. In properly fixed material it would be more possible to determine, in so far as one could histologically, whether these fishes retain a functional pronephros in the adult, as is the case in *Fierasfer*, *Zoarces* (Emery, 1880), *Lepadogaster* (Guitel, 1906) and *Mistichthys* (TeWinkel, 1935). The number of glomeruli in *Phenacostethus* is only slightly greater than that in *Mistichthys* (which has 12 to 13 on the average) and it would be of interest to discover whether the ratio of glomerular volume to body surface is in any way comparable in these two unrelated minute teleosts.

REPRODUCTIVE ORGANS

The ovary in the two species is single and lies along the anterior and ventral surface of the air bladder. In *Phenacostethus* there are on the

average fifteen maturing ova and the largest of these measure approximately $500\ \mu$. Each ovum is surrounded by a thick porous zona radiata with surface filaments forming, but the method of filament attachment was not observed. The ovary of *Gulaphallus* contains about sixty maturing ova. The oviduct, in both species continuous with the ovary and doubtless formed by fusion of the mesovarium, is thick-walled and consists of a deep epithelial lining (possibly pseudo-stratified columnar) and a well-developed circular layer of smooth muscle. Its exterior opening lies just anterior to that of the urinary duct.

The breeding habits and development of *Gulaphallus* have been described by Villadolid and Manacop (1934). They demonstrate that the fish is oviparous but that fertilization is internal. No sperm are present in the oviducts of either species examined by me; the *Gulaphallus* material is immature and it is possible that *Phenacostethus* was collected at a time which was not the breeding season.

The testis is an unpaired organ, on the aproctal side partly wrapped around the anterior wall of the air bladder (Fig. 2). Seminiferous tubules open antero-dorsally into a common collecting tubule. Because the structure of the ductus deferens differs considerably in the two species, each will be described separately.

In *Phenacostethus* all stages of spermatogenesis appear to be present in the testis. The ductus deferens is a long coiled tube, probably similar to that in *Phallostethus dunckeri* (Regan, 1913, 1916), forming a mass nearly equal to the testis in size. The tube is narrow in diameter for two or three coils, then increases to double its initial size, and finally extends into the priapium and into the genital papilla (Fig. 2). The epithelial lining of the tube is simple columnar, with tall cells in the narrower coils (Fig. 4), and cells about one-half this height in the large distal coils (Fig. 5), while that portion of the ductus deferens extending into the priapium is lined by low columnar epithelium. A valvular mechanism, greatly decreasing the lumen and lined by simple columnar epithelium, is present in the duct soon after it enters the genital papilla (Fig. 2). From this point the tube is slightly coiled, then dilates to form a seminal vesicle. Toward the tip of the papilla, the lumen is completely constricted and the wall seems to be composed of concentric layers of connective tissue, and very likely, some smooth muscle is also present.

The ductus deferens is crowded with spermatozoa in all specimens of *Phenacostethus* studied. In the narrow proximal coils of the tube (Fig. 4), there appear among the sperms small homogeneous globules which are, in all probability, secreted by the tall columnar epithelial cells of the lining. The size of these globules increases by one-half by

the time the large distal coils are reached (Fig. 5) and this increase may be the result of fusion of smaller globules. The staining capacity of these bodies varies: in Mallory's triple connective tissue stain, they are purplish in the proximal coils, but distally, brilliant red; in iron haematoxylin, brownish proximally, and distally gray or black; in alum haematoxylin and triosin, uniformly pink. These globules may be gelatinous spermatophores, but there seems to be no definite association between them and the sperms such as is described by Regan (1916) in two other phallostethids. Some spermatozoa may adhere to them, but there are quantities of free sperm in all parts of the duct. There is also a possibility that globules and sperm become united upon discharge, but study of living material is necessary to determine it.

The testis in the male specimen of *Gulaphallus* which was sectioned is large but immature. The ductus deferens, within the body cavity a very narrow duct probably without secretory function, dilates in the posterior portion of the priapium to form a seminal vesicle. The duct is of course entirely devoid of sperm so that the presence of sperm packets, affirmed by Bailey who evidently studied more mature specimens of this species, was not observed. (See Pl. 3, fig. 3 in Villadolid and Manacop, 1934).

Study of the two phallostethids described in the preceding pages, in spite of the handicap of poorly fixed material, has shown that, histologically, there are no striking differences in the viscera that may be attributed to the priapium or to the displacement of the organ outlets to the throat region. The persistence of the pronephros, which may be functional in the adults, is of general interest because it occurs so rarely in teleosts; it would be of value to examine other members of the group to determine whether that organ is always present in phallostethids. The great difference in the structure of the ductus deferens in the two species is undoubtedly related to family specialization. Possibly, the adult male *Gulaphallus* develops a glandular groove externally (as in *N. lankesteri*, Regan, 1916) to compensate for lack of abundant secretion on the part of the sperm duct; such a groove was not seen, however, in the pre-adult male examined histologically.

SUMMARY

The anatomy of the soft parts of two phallostethid fishes, *Phenacostethus smithi* and *Gulaphallus mirabilis*, has been studied.

1. Two groups of vestigial fin rays are present on the copulatory organs of *Gulaphallus* but are lacking on that of *Phenacostethus*.

2. A tuft-like pseudobranch, belonging to Granel's type II, is present in both species.

3. The viscera are located almost entirely anterior to the air bladder, and digestive, reproductive and excretory systems open externally on the sub-gular copulatory organ in the male and on the ventral surface of the gular region in the female. Liver, pancreas, spleen, thymus and thyroid are identified.

4. Neuromasts are scattered on the surface, and four canals, probably of the lateral line type, are present on the heads of both species.

5. The kidney contains one pair of pronephric glomeruli which may be functional. There are 13-18 mesonephric glomeruli in *Phenacostethus*, and 33-38 in *Gulaphallus*.

6. The ovary is single in both species and contains a relatively small number of ova. The oviduct, continuous with the ovary, is thick-walled and muscular.

7. The testis is a single mass. Seminiferous tubules open into a collecting tubule which continues as the ductus deferens. This duct forms a large coiled mass in *Phenacostethus* and is lined by simple columnar epithelium. Homogeneous globules, present among the sperms in the lumen, do not appear to form spermatophores. The ductus deferens in *Gulaphallus* is very narrow within the body cavity, but dilates to form a seminal vesicle in the copulatory organ.

LITERATURE CITED

- AURICH, H., 1937. Die Phallostethiden. *Mitt. der Wallacea Expedition, Zweite Reihe, Intern. Rev. ges. Hydrobiol. u. Hydrograph.*, **34**: 263-286.
- BAILEY, R. J., 1936. The osteology and relationships of the phallostethid fishes. *Jour. Morph.*, **59**: 453-483.
- DENNY, M., 1937. The lateral-line system of the teleost, *Fundulus heteroclitus*. *Jour. Comp. Neur.*, **68**: 49-65.
- EMERY, C., 1880. Fierasfer. *Atti della R. Accad. Lincei, Roma*, 3 ser., **7**: 167-254.
- GRANEL, F., 1927. La pseudobranchie des poissons. *Arch. d'Anat. micr.*, **23**: 175-317.
- GUI TEL, F., 1906. Recherches sur l'anatomie des reins des quelques gobiésocidés. *Arch. Zool. Expér. Gén. (Ser. 4)*, **5**: 505-700.
- HERRE, A. W., 1925. Two strange new fishes from Luzon. *Philippine Jour. Sci.*, **27**: 507-513.
- HERRE, A. W., 1926. Four new Philippine fishes. *Philippine Jour. Sci.*, **31**: 533-543.
- MYERS, GEO. S., 1928. The systematic position of the phallostethid fishes, with diagnosis of a new genus from Siam. *Am. Mus. Novitates*, No. 295, *Am. Mus. Nat. Hist., New York*.
- MYERS, GEO. S., 1935. A new phallostethid fish from Palawan. *Proc. Biol. Soc. Wash.*, **48**: 5-6.
- MYERS, GEO. S., 1937. Notes on phallostethid fishes. *Proc. U. S. Nat. Museum*, **84**: 137-143.
- REGAN, C. T., 1913. *Phallostethus dunckeri*, a remarkable new cyprinodont fish from Johore. *Ann. Mag. of Nat. Hist. (Ser. 8)*, **12**: 548-555.

- REGAN, C. T., 1916. The morphology of the cyprinodont fishes of the sub-family Phallostethinae, with descriptions of a new genus and two new species. *Proc. Zool. Soc. London*, 1: 1-26.
- SMITH, H. M., 1927. The fish *Neostethus* in Siam. *Science, N. S.*, **65**: 353-355.
- TEWINKEL, L. E., 1935. A study of *Mistichthys luzonensis* with special reference to conditions correlated with reduced size. *Jour. Morph.*, **58**: 463-535.
- VILLADOLID, D. V. AND P. R. MANACOP, 1934. The Philippine Phallostethidae, a description of a new species, and a report on the biology of *Gulaphallus mirabilis*, Herre. *Philippine Jour. Sci.*, **55**: 193-220.

REGENERATION OF GONAD TUBULES FOLLOWING
EXTIRPATION IN THE SEA-CUCUMBER,
THYONE BRIAREUS (LESUEUR)

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Several accounts infer that the extensive powers of visceral regeneration in the holothurians may extend to the gonads (Torelle, 1909; Deichmann, 1921; Bertolini, 1932) although they give neither precise data regarding the portion of the gonad involved nor even a gross analysis of the regenerated tissues. The present study considers the general problem of gonad regeneration in the holothurians with special reference to three specific points, namely, (1) the extent of the capacity to regenerate gonadal tissue, (2) the presence or absence of germ cells within regenerated tubules and (3) the possible origin of any germ cells found within them.

MATERIALS AND METHODS

Previous experience demonstrated (Kille, 1931) that *Thyone briareus* (Lesueur) is an extraordinarily good holothurian for experimental purposes. In contrast to the genus *Holothuria* which eviscerates through a tear in the cloaca, this genus casts off the entire anterior end of the body whenever it autotomizes the digestive system. With these parts lost, the animal is nothing but a dermo-muscular sac containing only a cloaca, respiratory trees, gonads, and mesentery. If left to itself, the circular body muscles at the extreme anterior end contract strongly so as to close off the body cavity from the sea. A healing process soon makes this closure permanent. However, if one makes a longitudinal cut at the anterior end equivalent to about one-fifth the length of the animal, many specimens may be turned inside out following their evisceration. In such a position the gonad tubules and the mesentery hang freely in the water. (Fig. 1, A, B, C). With the animal in this position, operations on the gonad are possible. Most specimens can then be turned right side out with little difficulty. If the animal is returned to running water, a high percentage survive the operation (Kille, 1937). In some cases the circular muscles of the body wall contract so

strongly that the animal cannot be turned right side out. Such individuals do not live more than a day or two.

THE REPRODUCTIVE SYSTEM IN *THYONE BRIAREUS* (LESUEUR)

A thick tuft of gonad tubules is found in each side of the dorsal mesentery as seen in Fig. 2. They originate from tissue localized within the mesentery in a region near the dorsal body wall (Fig. 2, gonad-basis) and about midway between the mouth and the anus (Fig. 1, C). These tubules empty their products into a common chamber located within the dorsal mesentery (gonad-basis chamber, Fig. 2). Anterior to the tubules this cavity continues as the lumen of a gonoduct which runs through the dorsal mesentery to open exteriorly at a point between the bases of the two dorsal tentacles. Each right and left half of the

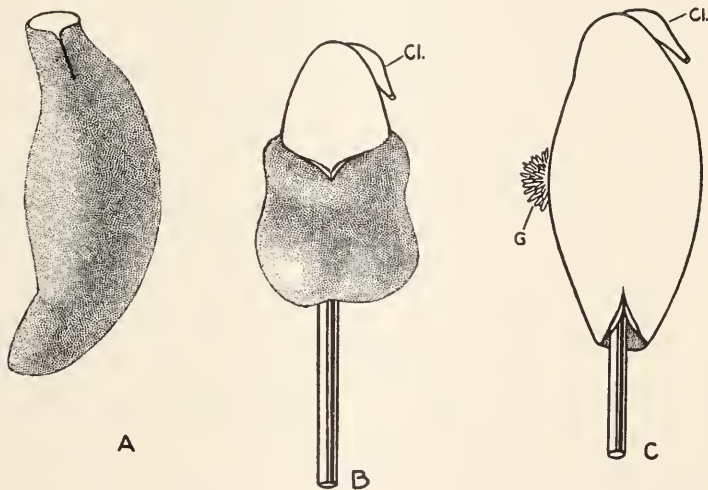


FIG. 1. *A*, *Thyone* immediately after evisceration with a short longitudinal slit through the body wall at the anterior end (ventral surface to the right); *B*, *Thyone* being turned inside out with the aid of a blunt glass rod; *C*, specimen turned completely inside out. *Cl*, cloaca; *G*, gonad. (Mesenteries and musculature of body-wall omitted.)

Thyone gonad consists then of (1) a lateral portion of a chamber located within the dorsal mesentery (the gonad-basis) and (2) a mass of tubules which take their origin from the ventro-lateral wall of the chamber (gonad tubule, Fig. 2). The right and left tufts of tubules are symmetrically developed, there being but little variation in the number and size of tubules between the two sides. Within each tuft, however, one can distinguish tubules in various stages of development. The ex-

treme anterior ones are small, unpigmented, translucent, immature tubules. Such a condition has been described for many genera (Théel, 1901; Mitsukuri, 1903; Haanen, 1914; Deichmann, 1930, etc.). Posterior to these is a group of tubules which grade into the slender, more mature tubules possessing pigmented, opaque walls, rounded extremities and greater length. These long, opaque tubules make up the bulk of the gonad, yet the shorter, anterior tubules comprise at least a fifth of the total number of tubules present. It should also be mentioned that

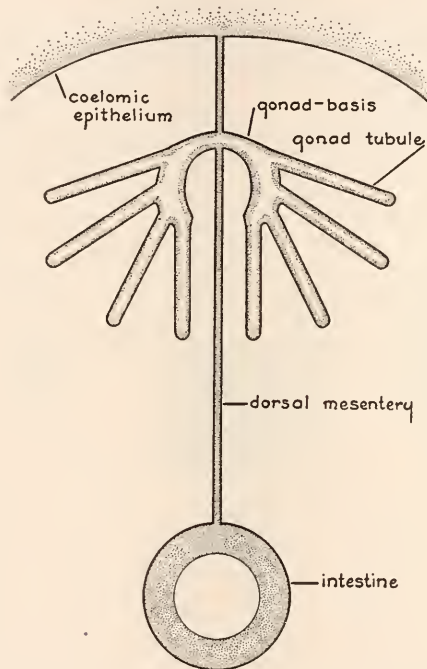


FIG. 2. Diagrammatic representation of a cross-section through a piece of the dorsal body wall with the attached mesentery to show the relationship existing between body wall, mesentery, gonad and intestine.

in the larger specimens collected during July and August there is an indication that posterior to the tuft of tubules still older tubules may have been lost.

In the medium-sized specimens used in these experiments, one can say that the larger the specimen, the greater is the mass of the gonad for the tubules are not only larger but their number is greater (see Table I). Within two larger specimens whose body volume was approximately 140 cc., the count for the female was 400 tubules while the male possessed 1035.

All these data indicate that there is a continued anterior proliferation of the tubules from the gonad-basis. Histological analysis of the tubules supports this conclusion. The posterior tubules show various stages in gametogenesis though there are fewer mature germ cells present than in those tubules more anterior, which may be packed with well-developed gametes. The most anterior tubules, however, show no ripe gametes but enormous numbers of germ cells in the early stages of gametogenesis. At the present time, I am not prepared to give an account of the seasonal variation in the gonad for all material discussed in this paper, whether normal or experimental, was collected in the months of July through October.

TABLE I

The total number of tubules found within a series of eleven specimens closely graded as to size.

Size of Specimens (Volume in cc.)	Approximate Number Tubules in Each Tuft	
	♀	♂
4	130	
4.5	110	
5.8	107	200
6.6	150	
8.0	160	
10.0		250
12.0	185	
13.0	170	
14.0	200	
19.0	170	

RESULTS OF THE REMOVAL OF THE GONAD TUBULES EITHER WITH OR WITHOUT THE GONAD-BASIS

Since it is possible that part of the gonad-basis is torn away with the gonad tubules whenever they are entangled in the digestive system during the process of evisceration, two types of operations were carried out. In Group I, the gonad tubules and the entire gonad-basis were extirpated from 6 female and 6 male specimens of medium-size. These were collected at Woods Hole in early August. In Group II, composed of a similar group of specimens, the gonad tubules were torn away from the gonad-basis by scraping the gonad area with the blunt back of a scalpel. This was done in order to produce a condition similar to that which occasionally exists after tubules have become entangled in a digestive system that is being eviscerated. Though this seldom happens in *Thyone briareus* (Lesueur) at this season, it frequently occurs in certain Holothuria when the gonads are well-developed.

In both groups, the ability to feed indicated that the new digestive system was established within 15 to 22 days, which is the average period of time required for unoperated specimens (Kille, 1935) as well as for the controls to these experiments. Evidently this unusual procedure for a coelomic operation has no injurious effect. None of Group I, however, showed any signs of gonad regeneration. Eleven specimens were killed for examination at various intervals after the operation, ranging from 46 to 129 days. The other remaining specimen of the

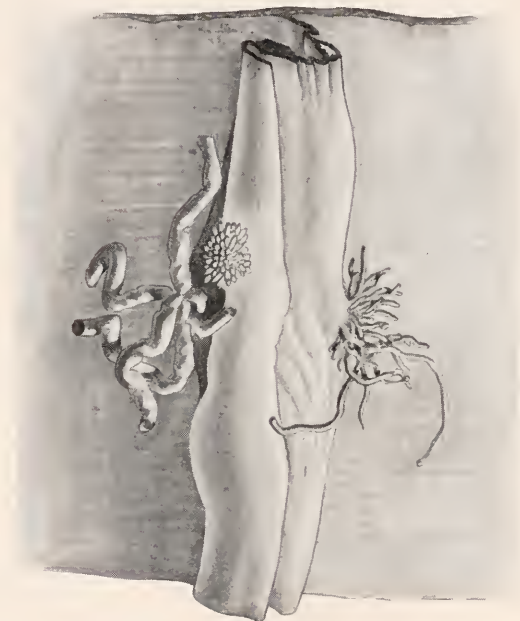


FIG. 3. Ventral view of the testes within a *Thyone* 63 days after the main mass of the gonad had been scraped away. For size comparison, the proximal quarters of 5 mature tubules of the original right testis are included on the reader's left.

group was in poor condition at the end of the experiment and was discarded. In all cases the new digestive system and its associated structures were well developed but the cut end of the gonoduct had healed blindly.

On the other hand, the gonad-basis of each specimen in Group II proliferated either a right, or a left, or a right and a left tuft of miniature tubules. Evidently all of the very smallest tubules of the original group were not always rubbed off for some of the newly proliferated groups showed a few tubules so much larger and longer than the rest

that it was clear that these belonged to the original gonad mass. Such groups were thus composed of a large number of newly proliferated tubules and, in addition, a smaller number of tubules which were present before the operation (Fig. 3, left gonad). This assumption is supported by the microscopic examination of a number of specimens which were killed immediately after the operation. In some cases, one could identify very minute tubules which remained attached to the remnant of the gonad-basis. At least this remnant must certainly remain even after the most violent self-eviscerations of holothurians.

The method of operation, however, frequently resulted in the removal of all the original tubules. Such an operation would easily account for those newly regenerated tufts which are like the right testis in Fig. 3. All the regenerated tubules are small and equal in development. This condition stands in sharp contrast to what is found in a normal male of equivalent size and season. Typically there would be only a fourth of this number of minute tubules and they would show a graded series as to length. Further evidence that these tubules arose at the same time is furnished by their histological similarities. In each tubule, the germ cells are packed rather solidly within the tubule. In contrast to this, the longer tubules as seen in the left testis (Fig. 3) possess a germinal epithelium surrounding a definite central lumen.

THE ORIGIN OF THE GERM CELLS WITHIN THE NEWLY PROLIFERATED TUBULES

Since the microscopic examination of the newly proliferated tubules showed that they were packed with cells which were identical cytologically with the primordial germ cells found within the tubules of the normal gonad, a careful study was made to determine the origin of these cells. Sections were prepared of normal gonads and of the gonad remnants left within operated but unregenerated specimens. Special attention was given to the anterior region of the normal gonad where the immature tubules occur. Here the small, club-shaped tubules appear to be diverticula of the wall of the gonad-basis. Anterior to these, one finds a few, minute, knobbed protuberances of the wall, each of which possesses a solid core of germ cells. Apparently, each core originates from an independent center within the wall of the gonad-basis, for just anterior to the protuberances separate nests of germ cells are found in those tissues. In view of their size and position, it is not surprising that they have never been described in this species. These nests of germ cells are evidently the equivalent of the "genital stolon" which is quite conspicuous in the dorsal mesentery of some holothurians (Théel, 1901 ;

MacBride, 1906) and from which the gonad-basis, gonad tubules and germ cells arise.

Examination of remnants of the gonad-basis left within operated animals shows that nests of germ cells may or may not be left on either side as a result of the operation performed upon Group II. This would seem to correlate with the fact that new tubules packed with germ cells may be proliferated on either or both sides following such operations. It is assumed then that such residual nests of germ cells are the source of the germ cells found within the newly proliferated tubules. Though none of these 12 animals failed to proliferate tubules on at least one side, one would expect this to occur occasionally if this assumption is true.

DISCUSSION

Since only a small part of the gonad-basis is ever lost when the gonad tubules are torn out during a process of visceral autotomy, these results show that an eviscerated holothurian does not merely mature certain small, residual tubules but may actually proliferate an entirely new set of gonad tubules. The removal of the original set of tubules is, in reality, a removal of only that part of the gonad in which a portion of the germ cells are maturing. The remnant of the gonad-basis possesses a store of primordial sex cells which are then contributed to the new tubules as they are proliferated at a rate probably four times the normal. It is a case similar to that reported in vertebrates where growth of accessory or residual gonadal tissue may follow incomplete gonadectomy. It is apparent, therefore, that true "regeneration of the gonad" does not occur but merely a proliferation of tubules from a growth zone or area of the gonad-basis.

In a period of 4 months, only a capacity for healing is demonstrated by the tissues of the gonoduct or of the gonad-basis when germ cells are absent. That the proliferation of tubules by the gonad-basis does not occur in the absence of germ cells is suggested by the facts that (1) a tubule stump (which contains no germ cells) never produces a new tubule, and (2) among the new proliferations, no sterile tubules have ever been found. It will also be noted in Fig. 3 that the tubules take their origin from an extremely localized area of the gonad-basis. This manner of origin suggests a dependence upon a restricted group of cells rather than upon a general capacity of cells widely distributed throughout the gonad-basis. It is of interest in this connection to note that in the chicken, Willier (1937) has obtained by means of chorio-allantoic grafting, male-like sex cords even though no primordial germ cells are present. A sexual gland without germ cells may also develop

in the urodele under certain conditions (Humphrey, 1928). Furthermore, Geigy (1931) has shown that in *Drosophila* the formation of a gonad is not dependent upon the presence of germ cells. By means of ultra-violet radiation he destroyed the primitive sex cells in the egg yet he obtained sterile ovaries and testes.

This dependent relationship of the origin of tubules upon the presence of germ cells in *Thyone* may be more apparent than real. A small group of cells at the anterior limit of the gonad area may retain a capacity for tubule formation whereas cells at more posterior levels do not. The relation then might be one of position only, the removal of the germ cells which are in this region invariably involving a loss of these tubule-forming cells. Microscopically there is no evidence for such a differential capacity among the cells of the gonad-basis. The only striking histological difference along its antero-posterior axis is the presence or absence of germ cells.

These results raise an interesting question relative to those holothurians such as *H. parvula* which undergo transverse fission in nature. In these species the gonad is located far anterior and therefore it is retained by the anterior portion. If the regenerative capacity of this genus is as limited as that of *Thyone*, then the posterior portions must continue their reproduction entirely by asexual means. While the occurrence of fission has frequently been reported in certain species of holothurians (Crozier, 1917; Deichmann, 1921; Kille, 1936), there has been no demonstration that it is the sole means of reproduction for certain individuals sterilized by the plane of a previous fission. Deichmann has reported (1921) "feebly developed" gonads within regenerating portions of *Holothuria* (*Actinopyga*) *difficilis*. In a recent communication to the author this investigator states that these gonads were seen only within anterior portions. There is then no report in the literature that the posterior portions ever develop a gonad. The absence of any such natural evidence for the regeneration of the gonads from other tissues coupled with the data obtained through this direct experimental approach makes a strong case for the segregation of tissues with germinal potencies within a highly restricted region of the dorsal mesentery.

As Stolte has recently (1936) pointed out, little is known concerning the cellular basis of regeneration within the echinoderm. Zirpolo (1928) believes there is a widespread totipotency among cells throughout the body of *Asterias*. On the other hand, studies on the genera *Stichopus*, *Holothuria* and *Thyone* leave us with only one established case to support such a generalization for Holothuria, namely, the formation of a new gut epithelium from the cells of the mesentery in *Stichopus regalis* (Bertolini, 1930). In all other instances new tissues apparently

arise from remnants of the old with the one possible exception of the anterior gut epithelium within a regenerating *Thyone* (Kille, 1935, p. 93). Since holothurians show an unusual capacity for regeneration there has been a tendency to assume that the tissues (or a tissue) of this group possess a totipotency throughout the entire animal. In light of the evidence, this assumption is unwarranted. Apart from the problem of organization, the unusual restorative powers are simply evidence of a normal capacity for cellular proliferation or migration and the maintenance of differentiation under new conditions.

SUMMARY

(1) The gonad in the sea-cucumber consists of two major portions: (a) a group of tubules in which germ cells are maturing and (b) a basal portion, the gonad-basis, from which these tubules arise. Failure to recognize this has probably led to the incorrect statements that these forms "will regenerate the gonad."

(2) In order to test the extent of the capacity to regenerate gonadal tissue, a partial or a complete gonadectomy was performed upon *Thyone briareus* (Lesueur). The gonad was exposed by turning the animal inside out after an induced autotomy of its digestive system and anterior end.

(3) That this procedure had no very injurious effects is shown by the fact that approximately 95 per cent of the operated animals survived. These and the controls regenerated the autotomized parts as rapidly as did eviscerated but unoperated animals.

(4) There was no regeneration of gonadal tissue following complete gonadectomy.

(5) If only the tubules were removed, new tubules might arise from a restricted, anterior region of the gonad-basis at a rate four times the normal (at this season).

(6) The only histological feature that distinguished this productive region from the rest of the gonad-basis was the presence of nests of germ cells.

(7) The possibility that tubule formation is dependent upon the presence of germ cells was suggested by still other facts. No sterile tubules were ever found among the regenerated tubules. Furthermore, the sterile proximal portion of a tubule which sometimes remained after a tubule had been torn out, did not give rise to a new tubule. Finally, when the tubules were roughly torn out, that portion of the gonad-basis containing the germ cells might or might not be lost and this is correlated with the fact that after such treatment, a *Thyone* may regenerate either a right or a left, or a right and a left tuft of tubules. It is

granted, however, that such evidence is not conclusive. Germ cell origin and tubule formation may be independently related to a totipotency possessed by only a few cells in a restricted portion of the gonad-basis for which these experiments provide no test.

LITERATURE CITED

- BERTOLINI, F., 1930. Rigenerazione dell' apparato digerente nello *Stichopus regalis*. *Publ. Stazione Zool. Napoli*, **10**: 439-447.
- BERTOLINI, F., 1932. Rigenerazione dell' apparato digerente nelle *Holothuria*. *Publ. Stazione Zool. Napoli*, **12**: 432-443.
- CROZIER, W. J., 1917. Multiplication by fission in *Holothurians*. *Am. Nat.*, **51**: 560-566.
- DEICHMANN, E., 1921. On some cases of multiplication by fission and of coalescence in *Holothurians* etc. *Vidensk. Medd. Dansk. Naturhist. Foren.*, **73**: 199-206.
- DEICHMANN, E., 1930. The *Holothurians* of the western part of the Atlantic Ocean. *Bull. Mus. Comp. Zool.*, **71**: 43-219.
- GEIGY, R., 1931. Action de l'ultra-violet sur le pôle germinale dans l'oeuf de *Drosophila melanogaster* (castration et mutabilité). *Revue Suisse de Zool.*, **38**: 189-288.
- HAANEN, W., 1914. Anatomische und histologische Studien an *Mesothuria intestinalis* Ascanius und Rathke. *Zeitschr. wiss. Zool.*, **109**: 185-255.
- HUMPHREY, R. R., 1928. The developmental potencies of the intermediate mesoderm of *Amblystoma* when transplanted into ventrolateral sites in other embryos: The primordial germ cells of such grafts and their rôle in the development of a gonad. *Anat. Rec.*, **40**: 67-101.
- KILLE, F. R., 1931. Induced autotomy in *Thyone*. *Science*, **74**: 396.
- KILLE, F. R., 1935. Regeneration in *Thyone briareus* Lesueur following induced autotomy. *Biol. Bull.*, **69**: 82-108.
- KILLE, F. R., 1936. Regeneration in *Holothurians*. *Annual Report Tortugas Laboratory, Carnegie Institution of Washington*, 1935-36: 85-86.
- KILLE, F. R., 1937. Regeneration of the gonad tubules in *Thyone briareus* Lesueur following extirpation. *Anat. Rec.*, **70** (Sup. 1): 133-134.
- MACBRIDE, E. W., 1906. *Echinodermata*. Cambridge Natural History. I: 567.
- MITSUKURI, K., 1903. Notes on the habits and life history of *Stichopus japonicus* Selenka. *Annot. Zool. Japan*, **5**: 1-21.
- STOLTE, H. A., 1936. Die Herkunft des Zellmaterials bei regenerativen Vorgängen der wirbellosen Tiere. *Biol. Rev.*, **11**: 1-48.
- THÉEL, H., 1901. A singular case of hermaphroditism in *Holothurids*. *Bihang. till K. Svenska Vet. Akad. Handlingar*, Afd. IV, **27**: 1-38.
- TORRELL, E., 1909. Regeneration in *Holothuria*. *Zool. Anzeig.*, **35**: 15-22.
- WILLIER, B. H., 1937. Experimentally produced sterile gonads and the problem of the origin of germ cells in the chick embryo. *Anat. Rec.*, **70**: 89-112.
- ZIRPOLO, G., 1928. Rigenerazioni eterotopiche in *Asterias tenuispina* Lmx. *Monit. zool. ital.*, **39**: 20-21.

EGG LAYING IN THE ACOELOUS TURBELLARIAN POLYCHOERUS CARMELENSIS¹

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The few accounts of egg laying in the Turbellaria Acoela are brief and unsatisfactory. The Acoela have one or two genital pores, except Nemertoderma (Steinböck, 1931). The absence of oviducts in the Acoela (Bresslau, 1933) leaves the question of egg emergence unsolved.

Von Graff (1905) concluded that the eggs of *Otocelis rubropunctata*, *Polychoerus caudatus* and *Convoluta roscoffensis* are liberated through the genital pore, which he considered the normal method for most Acoela. For species which possess no oviducts he agreed with Weldon, Sabussow and Monticelli that the mouth might serve in egg deposition. Yet von Graff pointed out (p. 1960) that oviducts had been described with reasonable certainty for only *Otocelis rubropunctata* and *Polychoerus caudatus* and that *Amphiscolops langerhansi* possesses no formed passage.

The evidence supporting von Graff's conclusions as to the rôle of oviducts and genital aperture was extremely meager. In his account of egg deposition in *Otocelis rubropunctata* (1904) he does not mention the path of emergence of the eggs. Gamble and Keeble (1903) stated that in *Convoluta roscoffensis* "frequently the body breaks in two across the opening of the oviduct . . .," i.e., presumably at the level of the genital pore. Gardiner (1895) is the only observer who has definitely stated that he witnessed the emergence of the eggs from the genital pore. He undoubtedly considered that the eggs of *Polychoerus caudatus* reach the female pore through the oviducts described and figured by Verrill (1893) and later by himself (1898). In these accounts the term oviduct was substituted for vitellarium, and the vitellaria were figured as uniting into a common duct leading directly to the female aperture. Mark (1892) found no connection between the vitellaria and the genital pore, and Löhner (1910) showed conclusively that this species possesses no oviducts. Löhner considered it probable that the eggs pass through a series of vacuoles in the marginal parenchyma and out by way of the female aperture.

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Subsequent references to egg emergence in the Acoela are equally inconclusive. Von Graff (1911) concluded with reference to *Anaperus gardineri* that the eggs probably leave by way of the mouth or through breaks in the body wall. With regard to the entire group Bresslau (1933, p. 118) stated that the eggs probably pass through "einfache Gewebslücken in das Zentralparenchym, um dann durch die Mundöffnung nach aussen befördert zu werden. Gelententlich vollzieht sich ihr Austritt noch direkter; durch Ruptur der Körperwand an irgendeiner Stelle." This latter alternative obtains in the case of *Polychoerus carmelensis*.

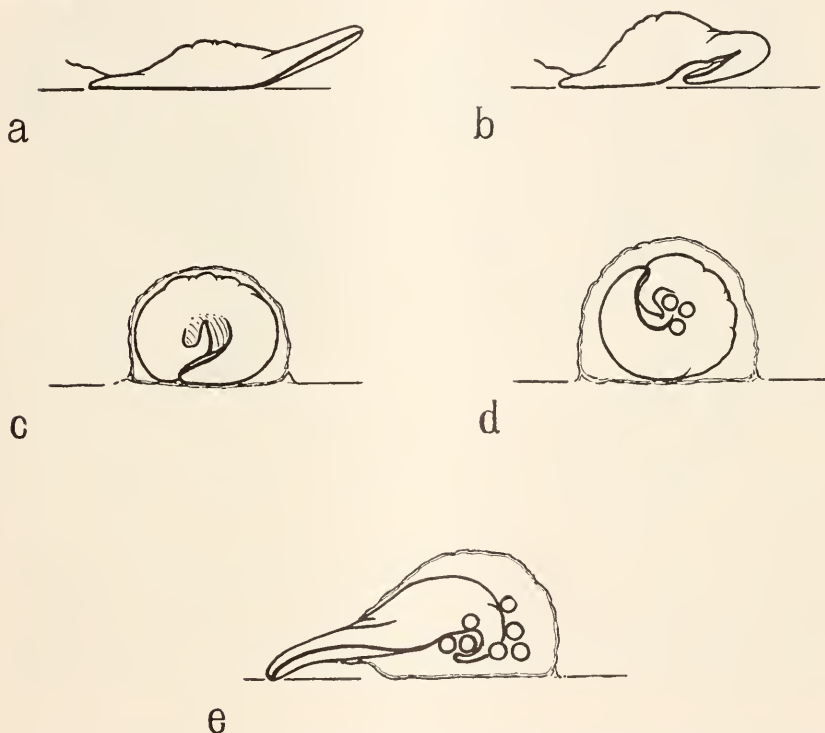


FIG. 1, a-e. Series of sketches to illustrate progressive stages of the process of egg laying.

MATERIAL AND METHODS

The reproductive system of *Polychoerus carmelensis* has been described and figured in a recent paper (Costello and Costello, 1938). Specimens about to deposit eggs were observed in situ by means of a hand lens or binocular microscope. Removal for examination invariably terminated the process. Twelve specimens were fixed during egg

laying, as follows: five in hot Heath's solution (50° to 60° C.), three in Lillie's, two in Worcester's (one "warm," one at 50° C.), one in Flemming's and one in Champy's solution. The subsequent study of hundreds of specimens has shown that hot fixatives are best for the general preservation of tissues. The specimens were sectioned individually and serially.

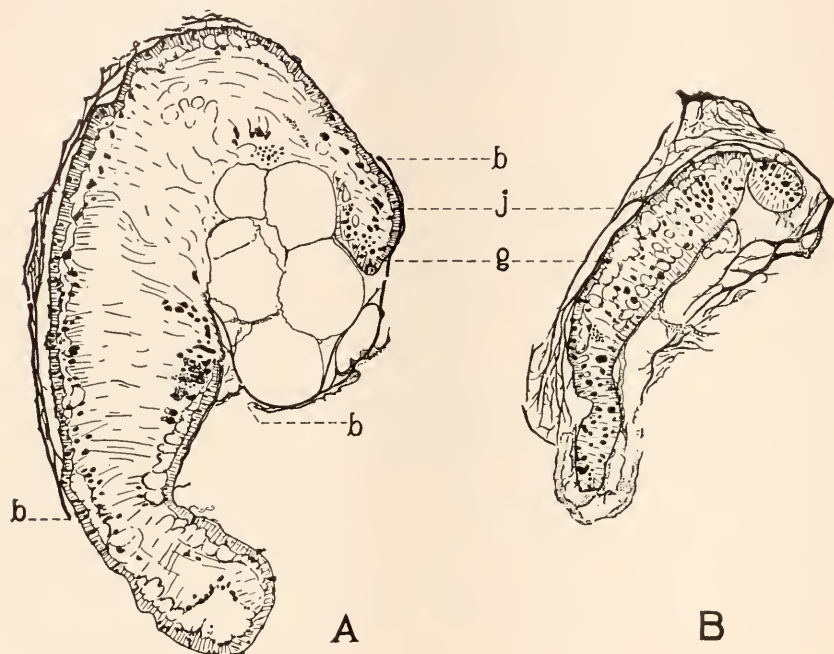


FIG. 2. Oblique sections of an egg-laying individual preserved in Lillie's fixative and stained by the Flemming tricolor method. *A*, through the side of the body; *B*, near the anterior end. To show jelly layers (*j*) of the egg mass, surrounding the worm, and the glands (*g*) which secrete the jelly. In *A*, the jelly is broken at several points (*b*); in *B*, the jelly mass is more complete. Magnification $43\times$.

OBSERVATIONS

An individual about to deposit eggs assumes a resting position on the substratum. The anterior end of the body is at first elevated (Fig. 1*a*), as the animal sways slowly from side to side, and is then bent ventrally in progressive stages (Fig. 1*b*). Meanwhile a jelly-like slime is secreted from the surface of the body and adheres to the substratum. As the anterior portion of the body continues the ventral curling, the posterior end relinquishes its attachment, and curls ventrally under the approaching anterior tip, forming a ball which is attached to the sub-

stratum only by the secreted jelly (Fig. 1c). During the curling, and subsequently, violent contractions are observable in the region of the vitellaria. The animal then begins to rotate, end over end, rather slowly within the gelatinous mass. During rotation, additional jelly is secreted by the surface of the animal (Fig. 2). Meanwhile, the eggs are emerging from the body of the animal, but are, for the most part, hidden within its folds (Fig. 1d). An occasional egg escapes laterally, and may then pass freely over the dorsal surface of the rotating animal (Fig. 5), within the jelly. Rotation continues for three or four minutes, after which the animal unrolls and works its way out of the jelly mass (Fig. 1e) through an opening so small as to greatly constrict the body. The eggs are left behind in the central cavity of the jelly mass, which contracts and forces the eggs into close proximity. The entire process of egg laying requires only five or six minutes.

Egg laying occurs infrequently during the early portion of the day, most frequently shortly after sunset (Table I). That egg laying occurs also during the early night is evidenced by large numbers of egg masses found in the morning in late cleavage stages.

The point of emergence of the eggs is concealed by the position assumed by the body during the process. Living individuals forcibly unfolded during egg emergence usually extruded no more eggs. In one case, however, an egg was observed to emerge through the ventral body wall at the level of the bursa.

The fixed material showed very clearly the position occupied by the mature eggs immediately before extrusion, and the point at which the eggs are shed, although no individual egg was fixed in the act of emerging. Of the twelve specimens, one had shed all its mature eggs, three had shed none, the others, a portion.

It has been noted (1938) that the oöcytes move dorsally within the vitellaria as they mature. However, eggs undergoing maturation occupy the greater part of the dorso-ventral thickness of the vitellarium. Immediately before deposition the mature eggs are located medially and ventrally between the body wall and the ventral surface of the bursa. The vitellaria are displaced laterally and the bursa is displaced dorsally, curving around the eggs as shown in Fig. 3. This arrangement was evidenced by the eleven fixed specimens which retained one or more mature eggs.

Evidence as to the point of emergence of the eggs is limited to the nine specimens which had shed one or more eggs at the time of fixation. There was no indication of the emergence of eggs by way of the mouth and intestinal parenchyma, nor by way of the vagina. Three of the five best preserved specimens showed perfectly definite points of rup-

TABLE I

*The numbers of egg depositions observed in the laboratory are listed according to the dates and hours of occurrence.
The hours of observation (—) on the respective dates are indicated.*

Date	A.M.						P.M.						Total							
	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-1	1-2	2-3	3-4	4-5		5-6	6-7	7-8	8-9	9-10	10-11	11-12
8/9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	2
10					—	—	—	—	—	—	—	—	—	—	4	3	1	—	—	8
11					—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1
12					—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2
17					—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1
19					—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
23					—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	2
24					—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2
26					—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	2
27					—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	1
Total										1	2	1	3	1	5	3	3			21

ture of the ventral integument in the region of the bursa, with the eggs in such a position as to indicate their emergence at this point.

The best two cases are illustrated by Figs. 4 and 5. In Fig. 4, the section passes through the flattened vagina (v), the female aperture (♀), the ventral protuberance in which two of the mature eggs appear, and

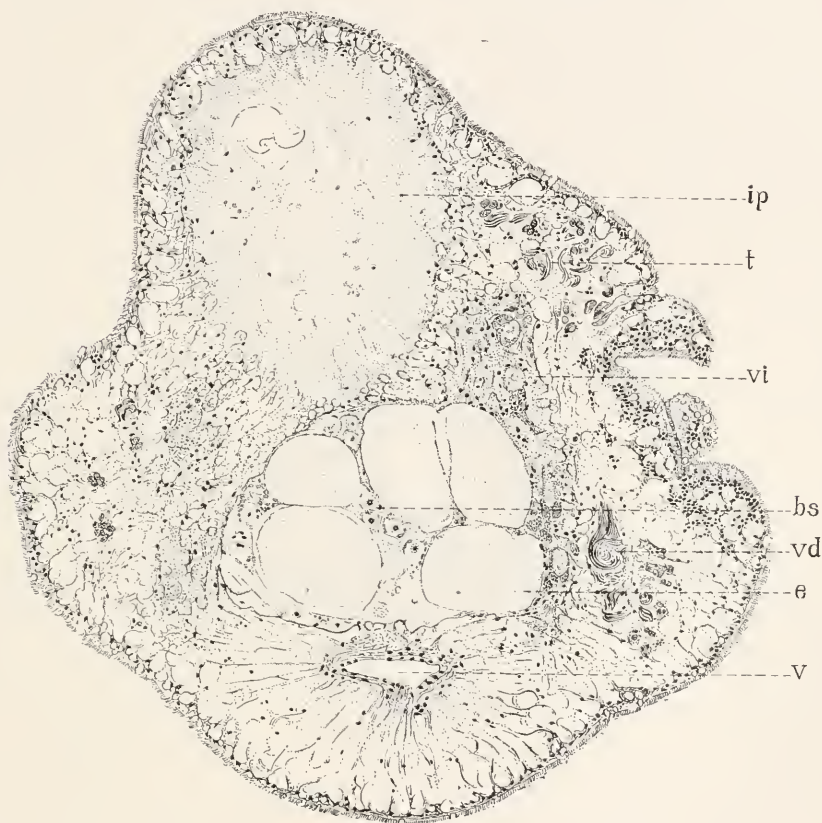


FIG. 3. Oblique frontal section of an egg-laying individual, to show the median ventral position of the mature eggs (e), and the displacement of the bursa (bs) around the eggs. Hot Heath fixation, stained in Heidenhain's haematoxylin. Magnification $72\times$. ip , intestinal parenchyma; t , testis follicles; v , vagina; vd , vas deferens; vi , vitellarium.

through the undisturbed mouth (m) and short pharynx (ph). The ventral integument shows a rupture (r) about 50 microns in width near one of the shed eggs (se). It is medial, extending through seven consecutive sections (70 microns). A jelly layer (j) surrounds the shed eggs and is connected with the ventral surface of the body near the point of rupture. The unshed mature eggs converge toward the point of



FIG. 4. Same individual as Fig. 3; twentieth section (0.20 mm.) ventral to that of Fig. 3. To show the rupture of the ventral integument (*r*), and the jelly strand connecting one of the two already shed eggs (*se*) with this point. The section passes obliquely frontally through the mid-region and nearly transversely through the mouth (*m*) and pharynx (*ph*). Magnification 50 $\times$. ♀, female genital aperture; *o*, immature egg; other abbreviations as above.

FIG. 5. Oblique sagittal section of an individual fixed after the extrusion of all of its eggs. To show the broken ventral integument and disrupted bursa, also the undisturbed mouth and intestinal parenchyma, and the flattened vagina. Hot Heath fixation, stained in Heidenhain's haematoxylin. Magnification 62 $\times$. Abbreviations as above.

rupture, as shown by the sections of the same individual represented in Figs. 3 and 4. The second specimen, represented in Fig. 5, shows the relation of the bursa, ruptured integument and shed eggs. In this case also the condition of the mouth and the extremely flattened vagina present striking evidence that these structures are not concerned in egg deposition. The ventral integument in the region of the bursa is disturbed and lacks cilia. Bits of tissue and jelly extend from the last shed egg to the region of the rupture. The bursa is greatly vacuolated, a portion of its tissue extending to, and apparently partially closing, the rupture. The disruption of the bursa results indubitably from the pressure of the eggs in their outward passage, combined with the violent muscular contractions observable in this region throughout the egg-laying process.

TABLE II

Distribution of the numbers of eggs per egg mass deposited in fingerbowls by animals collected 8/20/37, from the third to eighth day in the laboratory.

	Numbers of Eggs per Egg Mass					Total Numbers of Egg Masses
	1-5	6-10	11-15	16-20	21-25	
8/23.....	23	72	27	3	1	126
8/24.....	3	4	3	0	0	10
8/26*.....	14	34	19	2	0	69
8/27.....	3	13	19	5	0	40
8/28.....	5	18	12	2	0	37
Totals.....	48	141	80	12	1	282

* Two-day interval.

In the remaining seven specimens the exact point of rupture was not clear as a result of either the plane of sectioning, or of distortion following slow fixation. However, they correspond, in all essential features, with the two cases described above.

If *Polychoerus carmelensis* regularly discharges its eggs through rupture of the body wall, evidence of this should be observable in mature specimens fixed at random. Histological examination has shown frequent irregularities in the epidermis, parenchyma and bursa wall, including, in some cases, the interruption of epidermal ciliation. The general appearance suggests regeneration following recent disruption.

A few miscellaneous observations regarding the breeding habits of *Polychoerus carmelensis* in the laboratory and in the tide-pools may be here recorded. Animals brought into the laboratory and placed in finger-bowls do not, as a rule, begin to deposit eggs (or continue egg deposi-

tion) until the third day thereafter. During the following three days egg clusters are deposited in abundance, after which they become less numerous. The individual worms do not usually deposit all of their eggs at once, as has been suggested for the closely related *Polychoerus caudatus*. This is attested by the fact that the number of egg masses obtainable from a single dish may exceed the number of worms present. Data relating to the numbers of eggs per egg mass are given in Table II. The figures represent the numbers of egg masses deposited in five fingerbowls during the first eight days in the laboratory.

There appears to be a definite breeding season for this species in the Monterey region. Collections in 1937 were made at intervals from June 22 through July and August. In June no egg masses or young worms were found in the tide pools. By July 8 they were found in abundance on pebbles and shells and in the folds of the *Ulva*. Egg laying continued in the tide pools and aquaria through July and August, although in August there occurred an increasing number of apparently spent, large-sized individuals with reproductive system partially or completely absent. Thus, in 1937, the breeding season began in late June or early July and declined toward the end of August. Less extensive observations indicated that the same situation obtained during the season of 1936.

DISCUSSION

The foregoing account of egg laying in *Polychoerus carmelensis* provides evidence in support of the hypothesis of von Graff and Bresslau that the eggs of some of the Acoela may be discharged through breaks in the body wall. *Convoluta roscoffensis* is the only other species of this group in which egg laying has been described as involving rupture of the parent worm. In that species, however, the body of the worm is frequently broken completely in two at the time of egg deposition. The regenerative powers of the Acoela have been demonstrated by the experiments of Stevens and Boring (1905) and of Child (1907) for the Pacific Coast *Polychoerus*, and by Keil (1929) for the Atlantic Coast *Polychoerus caudatus*. The healing of injured surfaces occurs remarkably rapidly in *Polychoerus carmelensis*. For example, when worms are cut from the posterior end almost the entire length of the body, the two almost separate halves are quickly brought into contact by their normal locomotor activities, and heal within about ten minutes. A small notch may remain at the posterior end, but frequently all trace of the cut is obliterated. For organisms which heal and regenerate so readily the emergence of eggs through rupture of the body wall, in the absence of oviducts, may be regarded as a natural and direct method.

SUMMARY

The egg-laying habits of *Polychoerus carmelensis* are described, with especial emphasis upon the path of emergence of the eggs.

The mature eggs pass from the two vitellaria ventrally and medially between the ventral integument and the bursa seminalis, and emerge to the exterior through a median rupture at the level of the bursa.

The jelly mass is secreted by the entire surface of the animal during repeated rotation, head over tail. The eggs emerge during rotation, probably aided by violent muscular contractions observable at this time.

The entire process of egg laying requires five or six minutes.

LITERATURE CITED

- BRESSLAU, E., 1933. Turbellaria. *Kükenthal-Krumbach Handbuch der Zoölogie*, 2: 52-293.
- CHILD, C. M., 1907. The localization of different methods of form-regulation in *Polychoerus caudatus*. *Arch. f. Entw.-mech.*, 23: 227-248.
- COSTELLO, D. P., AND H. M. COSTELLO, 1937. Egg laying in the acoelous turbellarian *Polychoerus carmelensis*. (Abstract) *Anat. Rec.*, 70 (Suppl.): 138.
- COSTELLO, H. M., AND D. P. COSTELLO, 1938. Copulation in the acoelous turbellarian *Polychoerus carmelensis*. *Biol. Bull.*, 75: 85-98.
- GAMBLE, F. W., AND F. KEEBLE, 1903. The bionomics of *Convoluta roscoffensis*, with special reference to its green cells. *Quart. Jour. Mic. Sci.*, 47 (N. S.): 363-431.
- GARDINER, E. G., 1895. Early development of *Polychoerus caudatus*, Mark. *Jour. Morph.*, 11: 155-176.
- GARDINER, E. G., 1898. The growth of the ovum, formation of the polar bodies, and the fertilization in *Polychoerus caudatus*. *Jour. Morph.*, 15: 73-110.
- GRAFF, L. VON, 1904. Marine Turbellarien Orotavas und der Küsten Europas. I. Einleitung und Acoela. *Zeitschr. f. wiss. Zoöl.*, 78: 190-244.
- GRAFF, L. VON, 1905. Turbellaria, Erste Unterklasse: Acoela Ulj. H. G. Bronn, Klassen und Ordnungen des Tier-Reichs. Bd. 4, Lief. 68-74, S. 1902-1984. Leipzig.
- GRAFF, L. VON, 1911. Acoela, Rhabdocoela und Alloecoela des Ostens der Vereinigten Staaten von Amerika. *Zeitschr. f. wiss. Zoöl.*, 99: 1-108.
- KEIL, E. M., 1929. Regeneration in *Polychoerus caudatus* Mark. *Biol. Bull.*, 57: 225-244.
- LÖHNER, L., 1910. Untersuchungen über *Polychoerus caudatus* Mark. *Zeitschr. f. wiss. Zoöl.*, 95: 451-506.
- MARK, E. L., 1892. *Polychoerus caudatus* nov. gen. et nov. spec. Festschrift zum 70. Geburtstage R. Leuckarts. S. 298-309. Leipzig.
- STEINBÖCK, O., 1931. Ergebnisse einer von E. Reisinger und O. Steinböck mit Hilfe des Rask-Ørsted Fonds durchgeführten Reise in Grönland 1926. 2. *Nemertoderma bathycola* nov. gen. nov. spec. *Vidensk. Medd. fra. Dansk. Naturhist. Foren.*, 90: 47-82.
- STEVENS, N. M., AND A. M. BORING, 1905. Regeneration in *Polychoerus caudatus*. *Jour. Exper. Zool.*, 2: 335-346.
- VERRILL, A. E., 1893. Marine planarians of New England. *Trans. Conn. Acad.*, 8: 459-520.

THE EFFECT OF THE REMOVAL OF PERISARC ON REGENERATION IN TUBULARIA CROCEA

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INTRODUCTION

In 1903 Morgan observed that small pieces of *Tubularia* stems, which ordinarily regenerated hydranths at both ends, formed single hydranths at only one end when the other end of the stem was set upright in sand. Regardless of whether the distal or the proximal end was placed in the sand the exposed end was the one which formed the hydranth. Barth (1938*b*) extended these observations and showed that merely placing a glass capillary over the cut end of a stem would result in a complete inhibition of regeneration at the covered end. He also observed that when the coenosarc terminated a few millimeters below the level of the perisarc no regeneration occurred. It was suggested that oxygen lack was the probable cause for this inhibition, and that, in the last-mentioned case, the perisarc did not permit sufficient O₂ to reach the coenosarc for regeneration to occur. A logical experiment arising from these observations was one in which the perisarc was removed from a region along the length of the stem in order to expose the coenosarc to sea water. Morgan (1903) found that when long slivers of the stem of *Tubularia* (both perisarc and coenosarc) were cut out, the portions of the stem adjacent to this cut regenerated in the same way that two separated portions of a stem would regenerate, i.e., a hydranth formed at either side of the cut, with the oral ends facing the cut region. The two hydranths so formed were connected at the oral ends during the initial stages of regeneration but separated after a while. Goetsch (1929) briefly reported that when all cut ends of a stem of *Pennaria* were sealed with wax, the removal of some of the perisarc was sufficient stimulus to cause regeneration at the exposed region. Peebles (1931) attempted to get lateral outgrowths from stems of *Tubularia* by "wounding the perisarc" but failed to do so. The present work was an attempt to work out in some detail the consequences of exposing the coenosarc in *Tubularia*. These experiments were performed in a preliminary way in the summer of 1937 (when it was found

that exposing the lateral coenosarc did result in hydranth formation, especially if the ends were ligatured) and were repeated and quantitative data gathered in the summer of 1938. I should like, at this point, to thank Professor Barth for his many suggestions which were so helpful throughout the course of these experiments.

METHOD

Essentially the same technique as the one described by Barth (1938a) was used for selecting the stems, keeping them, and for performing the necessary calculations. Straight, unbranched stems were selected from colonies of long (8-9 cm.) stems. These were selected for uniformity in thickness, etc. The hydranth plus 3-5 mm. of the stem was removed and the required length of stem was cut from the region immediately proximal to this. The ends were then ligatured as desired and the perisarc was removed. This removal was effected by means of a pair of iridectomy scissors used under a binocular microscope. Ligatured stems were selected at random for the various groups in an experiment. The desired measurements (the area of the exposed coenosarc and the diameter of the stem) were made with an ocular micrometer. The stems were kept on a gauze platform in a dish where aerated sea water was circulating constantly. To insure uniformity of conditions all of the stems involved in any one experiment were kept together in the same dish. The rate of regeneration was obtained by measuring the length of the regenerant at the time it was constricted from the rest of the stem, and employing the time required for this to occur as t_1 in the formula $R = \pi r^2 L / t_1$ (see Barth, 1938a) where r is the radius of the stem at the region involved, and L is the length of the primordium. In cases (Fig. 1—1 C) where the regenerant was not a complete cylinder the volume was obtained indirectly by employing a bit of rubber tubing as a model for the stem. A sector of the tubing equivalent in size and shape to the regenerant was cut out and weighed. It was found that this was from one-third to one-half the total weight of the tubing. The volume of the actual cylinder of stem was calculated using the longest length of the regenerant as L , and then the necessary one-third or one-half was subtracted from this figure. All of the data presented are averages for the various groups considered. Only stems showing regeneration within 72 hours after the beginning of the experiment were considered in the calculations.

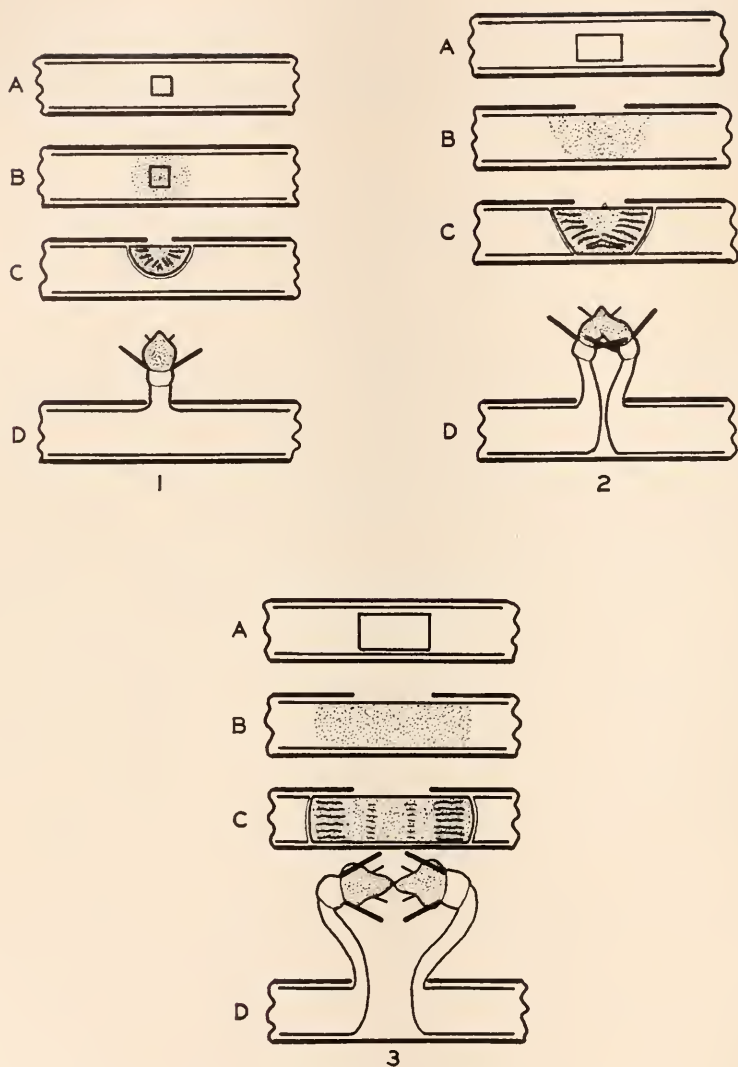


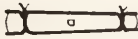
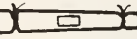
FIG. 1. Diagrams to show the effect of increasing the size of the exposed area upon the morphology of the regenerants. One, 2, and 3 are the three size groups. *A* indicates the amount of perisarc removed. *B* shows the beginning of pigment deposition. *C* shows the constriction of the regenerant from the rest of the stem and the appearance of the tentacle buds. *D* represents the condition of the regenerant after it has emerged.

RELATION BETWEEN THE AREA EXPOSED AND THE RATE OF REGENERATION

After preliminary experiments indicated that the exposure of the coenosarc was a sufficient stimulus for regeneration, an attempt was made to see whether there was any relation between the area exposed and the rate of regeneration. If, as was suspected, exposure to oxygen was the cause for regeneration a greater amount of exposed tissue should result in a larger regenerant. This was found to be the case. Table I presents data from two experiments which illustrate this. Ex-

TABLE I

Fifteen-millimeter stems. *A*, *B* and *C* are the three size-groups for the areas exposed. *No.*, number of stems used; *Diam.*, diameter in micra; *Area* in square micra; *T_i*, time in hours; *No. Reg.*, number of regenerants; *Rate*, rate of regeneration.

	A	B	C
			
EXP. 1			
NO.	10	9	10
DIAM.	769	789	802
AREA	55 073	153 793	281 559
T _i	43.7	48.1	47
NO. REG.	4	8	9
RATE	36	76	137
EXP. 2			
NO.	10	10	10
DIAM.	457	490	477
AREA	48 152	132 793	164 962
T _i	38.5	38.6	32.4
NO. REG.	7	8	9
RATE	71	89	129

periment 1 was performed early in June when the stems were thick and experiment 2 was a repetition of the experiment performed in July when the young thin stems prevailed. The results are essentially the same as far as rate of regeneration is concerned. As the area of the exposed region was increased, the rate of regeneration went up. Since the average time in which this regeneration occurred was approximately the same for all of the groups in any one experiment, it can be safely assumed that it was the amount of tissue converted in a unit of time that was increased as more coenosarc was exposed. (See Barth, 1938*b*, where both t_1 and L vary with the oxygen tension.)

PLATE I



1. A central regenerant (as in Fig. 1—1C) at the constriction stage.
2. A central regenerant (as in Fig. 1—3C) before constriction. The tissue is slightly contracted.
- 3 and 4. Single hydranths (as in Fig. 1—1) after emergence.
- 5—12. Various degrees of fusion of the two emerged hydranths. (10 was cut from the stem at the point of emergence.)
13. Looking down on two complete hydranths which are connected at the hypostomes.

(Figs. 2 and 13 were photographed by Dr. A. N. Solberg.) (The author wishes to thank Mr. Jack Godrich for his assistance in preparing the graphic material for publication.)

MORPHOLOGICAL AND POLARITY VARIATIONS

When thicker stems were used (Table I, Experiment 1 and preliminary) in the above experiment it was found that in addition to a variation in the rate of regeneration there was a variation in the morphology and in the polarity of the regenerants which depended roughly on the amount of tissue exposed. As depicted in Fig. 1 and in Plate I, there were three main categories of regenerants. When the exposed area was small (Fig. 1, case 1; Pl. I, 1, 3 and 4), only a limited amount of the coenosarc immediately surrounding it was involved in regeneration. In these cases the tissue involved in hydranth formation did not extend completely around the stem but formed a curved disc. When the proximal tentacle buds appeared they radiated out from the exposed central area. The distal tentacles frequently formed during the later stages of regeneration or during emergence. Such regenerants emerged as complete single hydranths whose polarity, i.e., oral-basal axis, was at right angles to the original polarity of the stem. In most of the cases where a greater amount of coenosarc was exposed (Fig. 1, case 2) the tissue involved in regeneration extended completely around the stem but was not symmetrical. A longer portion of the stem was involved in hydranth formation in regions immediately adjacent to the opening, while less and less of the stem was involved the farther it was removed from the opening. Such regenerants formed cylinders with oblique ends and had the appearance of trapezoids when they were viewed from the side (Fig. 1, case 2, *B* and *C*). These emerged as two more or less completely fused hydranths (Pl. I, 5 to 13). The extent of the fusion depended on the length of the region farthest from the opening. Where this was short the two hydranths were completely fused, as this increased in size the bases of the hydranths became separate but the oral portions were in common. All degrees of such fusion were encountered. The oral-basal axis in these cases was at various oblique angles to the original axis. In a few cases very large areas were exposed (the perisarc was removed from about one-half the surface of the stem for a length of 2-3 mm.) and a complete cylinder of coenosarc was involved in hydranth formation. The tentacle buds formed symmetrically around the stem (Fig. 1, case 3, *C*) and when the two hydranths emerged they were complete and mirror-images of each other. They remained fused at the hypostomal region and did not separate even when kept for 10 days. Here the original polarity was retained by the proximal regenerant and was completely reversed in the distal one. These results were similar to Morgan's cases (cited above) except that in his experiments the two hydranths separated. This difference is probably due to a mechanical factor, i.e., in Morgan's cases there was a thin bridge of tissue between

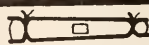
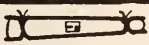
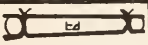
the two hydranths and this probably broke very easily when the regenerants contracted, while in the present cases there was a thicker connection which was not broken.

INJURY TO TISSUE

In the light of Child's (1927*b*) experiments on *Corymorpha*, where laceration of the tissue (in this case not covered by a perisarc) was sufficient to cause regeneration, the work described above was open to the valid criticism that injury to the tissue and not exposure to the sea water was the stimulus for regeneration. The following experiment was performed to settle this question: Three groups of stems (Table II)

TABLE II

Fifteen-millimeter stems. *A* is the group in which the perisarc was removed without injury to the coenosarc. *B* is the group in which the perisarc was removed and the coenosarc was lacerated. *C* is the group where the coenosarc was lacerated and where the perisarc was replaced. Legend as in Table I.

	A	B	C
			
NO.	9	10	10
DIAM.	725	745	738
AREA	189488	177805	187638
T _i	45.5	47.4	—
NO. REG.	6	5	0
RATE	64.6	67.5	0

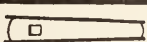
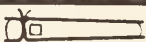
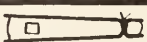
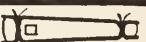
were selected and ligatured at both ends. The perisarc was removed very carefully in the first group and whenever there was any suspicion that coenosarc had been injured the stem was discarded. In the second group the coenosarc was deliberately lacerated after the perisarc had been removed. In the third group a three-sided flap was made in the perisarc and the underlying coenosarc was lacerated after the flap had been deflected. The flap of perisarc was then allowed to fall back into position—covering the lacerated tissue. Table II shows that the first two groups regenerated at approximately the same rates, while the third group did not regenerate at all. This shows conclusively that exposure to sea water is, in itself, a sufficient stimulus for the initiation of regeneration.

DOMINANCE RELATIONS

Since all of the experiments described above were performed on stems which were ligatured at both ends an attempt was made to see whether the open ends of a stem would affect the regeneration of an exposed region of that stem. Three experiments were performed in each of which four groups of 20 mm. stems were set up. In group A both ends were left undisturbed; in group B the distal 2-3 mm. were ligatured so that there was no continuity between that region and the rest of the coenosarc; in group C the proximal 2-3 mm. were ligatured; and in group D both ends were ligatured. Perisarc was then removed from the stem at various levels. In Table III the data for an experiment in

TABLE III

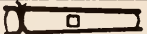
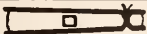
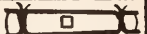
Twenty-millimeter stems. Showing the effects of the ends on a central regenerant. The openings indicated were 5 mm. from the distal ends. A, both ends open; B, the distal end ligatured; C, the proximal end tied off; D, both ends ligatured. Legend as in Table I.

	A	B	C	D
				
NO.	9	9	10	10
DIAM.	659	682	675	708
AREA	170053	145365	157562	192508
T _i	-	36.3	34.3	39
NO. REG.	0	3	3	4
RATE	0	57.7	34.9	115.5

which the perisarc was removed from a region 5 mm. from the distal end is presented. Table IV contains the data for an experiment in which the perisarc was removed from a region which was 10 mm. from the distal end; and Table V contains the data when the perisarc was removed from a region which was 15 mm. from the distal end. It is apparent, when these data are examined, that the ends, especially the distal end, exert a strong inhibiting influence (dominance) on the central regenerant when these ends are in continuity with the tissue of the stem. When this continuity is broken by a ligature the inhibition is no longer present and the rate of regeneration of the central region increases greatly. The proximal end by itself does not exert nearly as much of an inhibiting influence as does the distal. In Tables IV and V it can be seen that when only the distal end was tied off the rate of regeneration was much higher than when the proximal end was tied off.

TABLE IV

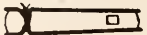
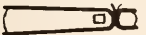
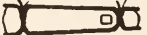
Twenty-millimeter stems. The opening is 10 mm. from the distal end. Rest of table as in Table III.

	A	B	C	D
				
NO.	10	9	10	9
DIAM.	632	586	603	597
AREA	184778	179731	165931	139379
T _i	44	56.6	52	50
NO.REG.	2	5	2	7
RATE	9.1	42.8	8.9	95.6

In fact the distal end exerted just about as much dominance by itself, in these cases, as did both ends combined. C in Tables IV and V is as low as A. In all three experiments there is a marked increase in the rate of regeneration when the inhibiting effect of both ends is removed. The one group which is in apparent discord with these data is group C in Table III. Here, despite the fact that the distal end is not liga-

TABLE V

Twenty-millimeter stems. The opening is 15 mm. from the distal end. Rest of table as in Table III.

	A	B	C	D
				
NO.	10	10	10	9
DIAM.	553	512	541	514
AREA	142567	117155	161352	111024
T _i	—	44.5	—	47.6
NO.REG.	0	6	0	9
RATE	0	39.3	0	68.9

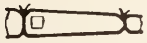
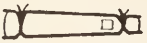
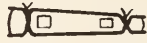
tured, the rate of regeneration of the central region is fairly high. This is probably due to the fact that the perisarc was removed from a region which was fairly close to the distal end (appr. 5 mm.). The same factors responsible for the appearance of bipolar regenerants in small pieces of *Tubularia* are probably responsible for the high rate of regeneration of the central regenerants in this group. This has been explained (Morgan, 1901) as being due to the fact that these tissues are very

close together and are probably metabolizing at about the same level and thus do not inhibit each other. Whether or not this is the case, the evidence still indicates definitely that the ligaturing of the ends will result in a great increase in the rate of regeneration of a central exposed region and that the ends do exert a dominance over a central region when they retain continuity with such a region.

Finally, an experiment was performed to determine whether dominance was exerted by a more distally placed exposed region over a more proximally placed exposed region. In Table VI we can see that

TABLE VI

Fifteen-millimeter stems. Both ends ligatured. *A*, the exposed region at the distal part of the stem; *B*, the exposed region at the proximal end of the stem; *C*, an exposed region at both ends. Legend as in Table I.

	A	B	C	
				
NO.	10	10	D 10	P
DIAM.	503	514	521	538
AREA	149021	140021	163738	173309
T _i	42.4	48.6	44.7	—
NO. REG.	5	6	7	0
RATE	41.3	45.9	60.2	0

this is definitely the case. In this experiment 15 mm. stems were used and both ends were ligatured close to the cut surfaces. The two exposed regions were separated by about 10 mm. The distal exposed area, though slightly smaller, completely inhibited the proximal one. Groups *A* and *B* in this table serve as the controls.

DISCUSSION

For the first few hours after its exposure the coenosarc of *Tubularia* is in a condition approximating the early indeterminate stages of an embryo (e.g., the ectoderm of an amphibian embryo). There is a tendency to continue its usual vegetative activity and produce more perisarc to replace that which has been removed. Sooner or later, however, what seems to be a competition between the tendency to retain the vegetative state and the tendency to form hydranth material begins to manifest itself. A visible evidence of this is the appearance of the pink pigment which typifies the first stage in regeneration. Now, de-

pending on the existing conditions (size of the opening, dominance of another region, or inherent conditions within the stem), the coenosarc either becomes definitely determined to form hydranth tissue or retains its original condition. In the former case a hydranth forms and eventually breaks through the thin perisarc which had been secreted in the earlier stages. In the latter the pigment disappears after a while and the coenosarc continues to produce more perisarc until the site of the removal cannot be distinguished from its surroundings. Occasionally, when the opening is very small or when another region is dominating it, the exposed coenosarc does not even begin to regenerate (i.e., no pink pigment is deposited) but continues to form more perisarc as though there had been no interruption. The analogy to amphibian material is made more striking when it is shown that there is a period after which the determination of the hydranth-forming material becomes irreversible. This has been shown by Peebles (1931) and by another method by the author in some unpublished preliminary work. The analogy breaks down, however, when the stimulus for the determination is considered. Although the primary stimulus for form and organ formation in amphibian embryos may come from an outside source, the work of the past few decades has indicated that the immediate cause of determination of a given structure comes from adjacent tissues. In *Tubularia*, as well as in many other hydroids (Child, 1927*a*) the stimulus for hydranth formation comes from outside of the organism. (The various parts of the hydranth may be determined as in the *Amphibia*). Many external agents have been cited (Child, 1929) as the stimulators for regeneration.

In referring to the experiments in which stems of *Tubularia* were either placed end down in sand or in which the end was capped with a glass capillary, both Morgan and Barth suggested that an oxygen lack might be responsible for the ensuing inhibition. It was implied that the presence of sufficient O_2 would allow regeneration to proceed. Accumulated wastes rather than O_2 lack was not excluded as a possible cause of the inhibition. The present experiments do exclude this since the sea water in which the stems were kept was well aerated and was kept in constant circulation (the glass-capping experiments were repeated under these conditions and the same results were obtained). It seems likely, therefore, that the inhibition of regeneration is due to oxygen lack; and that when an adequate supply of O_2 reaches the tissue it serves as a stimulus for regeneration. This idea is rendered more plausible when the effects of varying the oxygen tension on the rate of regeneration are considered. Barth (1938*b*) found that both the length of the regenerant increased and that the time in which constriction of the re-

generant occurred decreased as the oxygen tension was increased. This indicates that as the only variable in these experiments, i.e., oxygen, was increased a greater amount of tissue was converted into hydranth tissue in each unit of time. This bears a definite similarity to the experiments cited above in which the size of the regenerant increased when more coenosarc was exposed to the influence of the sea water and suggests a similar causal relationship. The similarity between the action of oxygen and the exposure of the coenosarc is made more apparent by some unpublished work of Dr. J. A. Miller. (Dr. Miller was kind enough to grant the author permission to mention some of his material in relation with the present work.) This work of Miller's concerns the effects obtained with varying the oxygen tension of the sea water in which small bits of *Tubularia* stem were kept. These small stems ordinarily yield bipolar regenerants. As the tension of the oxygen was increased, the number of incomplete bipolars was increased. This indicates that the various regions of the regenerant increased in size and that the central portion of the small stem was used for more distal structures instead of for the more basal regions which they would have formed in a lower concentration of oxygen. Since this increase in primordia size occurred equally at both ends, there was not enough material to form complete hydranths and two symmetrical incomplete forms resulted—more or less of the basal regions were missing. These results are, to some extent, the converse of the results obtained when the perisarc was removed from the stem. When the area exposed was small the regenerant was small and a single complete hydranth formed,—just as the bipolars were usually small complete hydranths when the oxygen tension was low. When the openings were sufficiently large more tissue was involved and the regenerants were enlarged and were more complete. When Miller increased the oxygen tension the bipolars became more incomplete for the same reason, i.e., the regenerants became enlarged but at the expense of their basal regions. The striking similarity between the effects of exposing the coenosarc and increasing the oxygen tension leads one to conclude that the former is also probably the effect of the oxygen in the sea water. The experiment where the perisarc was replaced helps to substantiate this.

If the premise that an increased oxygen supply stimulates the coenosarc to form hydranth tissue is granted, then the results obtained in these experiments can be explained very readily. The coenosarc must be metabolizing at a certain level before hydranth tissue will form. When a small opening is made in the perisarc only the tissues immediately around the opening get enough O_2 to raise them to this level. Since the basal portions of the hydranth (including the proximal ten-

tacles) form at the lower level of activity, and since this lower level is symmetrical around the opening, a small but complete hydranth forms. But, since the region exposed to the oxygen is at the side, and the high activity level is around this, the new hydranth's polarity is at right angles to the polarity of the stem. The other form and polarity variations may be explained in the same way. As more oxygen is admitted into the stem, more of the coenosarc is activated to form hydranth tissue and, depending on the resulting gradient of oxygen, the two hydranths produced are more or less fused. If a small region of the stem opposite the opening is activated the basal regions as well as the distal will be fused. If a greater region of this part of the stem is activated the basal portions of the hydranth will be separate. And as this region increases in length more and more of the hydranths become separated until two complete forms are produced.

The work on dominance does not throw any new light on the factors involved in this phenomenon, but simply illustrates the inhibition exerted by the ends upon a central region.

SUMMARY

1. Experiments in which lateral regions of the coenosarc of *Tubularia* were exposed indicate that the rate of regeneration in such regions is proportional to the area exposed to the influence of freely circulating sea water.

2. The morphology and polarity of central regenerants in thicker stems varies with the area exposed. Forms ranging from single complete hydranths, whose polarity was at right angles to the original polarity, to two hydranths, fused to a greater or lesser extent, were obtained. The polarity of the double hydranths varied with the degree of fusion. All angles to the original polarity were obtained.

3. Injury to the exposed coenosarc is not a factor in the present cases.

4. The ends of a stem exert a definite dominance over the exposed central regions. This dominance may be removed by ligaturing the ends. When this is done the rate of regeneration of the central region increases greatly.

5. A more distally placed central exposed region inhibits another exposed region placed more proximally on the same stem.

6. It is suggested that the probable stimulator of regeneration in *Tubularia* is the oxygen in the sea water. The results on rate of regeneration and on morphological and polarity variations may be explained by the amount of oxygen available to the coenosarc when areas of different size are exposed.

LITERATURE CITED

- BARTH, L. G., 1938a. Quantitative studies of the factors governing the rate of regeneration in Tubularia. *Biol. Bull.*, **74**: 155.
- BARTH, L. G., 1938b. Oxygen as a controlling factor in the regeneration of Tubularia. *Physiol. Zool.*, **11**: 179.
- CHILD, C. M., 1927a. Modification of polarity and symmetry in *Corymorpha palma* by means of inhibiting conditions and differential exposure. *Jour. Exper. Zool.*, **47**: 343.
- CHILD, C. M., 1927b. Experimental localization of new axes in *Corymorpha* without obliteration of the original polarity. *Biol. Bull.*, **53**: 469.
- CHILD, C. M., 1929. Physiological dominance and physiological isolation in development and reconstitution. *Arch. f. Entw.-mech.*, **117**: 21.
- GOETSCH, W., 1929. Das Regenerationsmaterial und seine experimentelle Beeinflussung. *Arch. f. Entw.-mech.*, **117**: 211.
- MORGAN, T. H., 1901. Further experiments on the regeneration of Tubularia. *Arch. f. Entw.-mech.*, **13**: 528.
- MORGAN, T. H., 1903. Some factors in the regeneration of Tubularia. *Arch. f. Entw.-mech.*, **16**: 125.
- PEEBLES, F., 1931. Some growth-regulating factors in Tubularia. *Physiol. Zool.*, **4**: 1.

THE RÔLE OF TEMPERATURE IN HYDRANTH FORMATION IN TUBULARIA

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Marine Biological Laboratory, Woods Hole)

Tubularia crocea (Agassiz) is a hydroid found on wharfs and piles in the Woods Hole region. It is abundant until the last part of July when the hydranths are dropped and the coenosarc retreats into the perisarc. The colonies remain in this condition until October when growth begins anew. The experiments reported in this paper show that temperature is an important factor in regulating this periodicity.

Autotomy and resorption of hydranths under a variety of conditions have been studied by a number of investigators, for example, Bäschlin (1932), Cerfontaine (1903), Gast and Godlewski (1903), Godlewski (1904), Huxley and deBeer (1923), Loeb (1900), Morse (1909), Riddle (1911), and Thatcher (1903). Morse (1909) made a special study of the cause of autotomy of the hydranth in *Tubularia* and concluded that "... temperature seems to be the only consistent factor involved in the decapitation. When the temperature is kept at about 10° C. or 15° C., the hydranths are retained, regardless of any other factors with the one exception of lack of oxygen, which we believe to be inoperative except under wholly artificial conditions." Sumner, Osburn and Cole (1913) recorded the disappearance of *Tubularia crocea* during the summer from the harbor at Woods Hole but noticed that in cooler waters the colonies may remain in an active condition throughout this period. Elmhirst (1922) kept colonies of *Tubularia* in his aquarium for three years and noticed that hydranths appeared in midwinter and were lost in midsummer. Light and oxygen were thought to be the principal factors involved.

If high environmental temperatures cause *Tubularia* to exist in a dormant condition, then keeping them in colder water should result in renewed growth. It was also thought that oxygen might be a factor since its importance in regeneration has been demonstrated by Barth (1937, 1938) and Miller (1937). The following experiments were performed to test the action of temperature and oxygen on dormant colonies of *Tubularia*.

EXPERIMENTS

On August 6, 1938 an experiment was begun to test the effect of oxygen and temperature on dormant colonies. Two 500 cc. flasks were placed in an 18.4° constant temperature bath. Into one of these, *A*, air was bubbled continuously at the rate of 1 liter per minute. The other, *B*, received oxygen at the rate of 1 liter per 11–14 minutes. These gases kept the water saturated and in rapid motion. Two similar flasks were placed in running salt water which varied from 23.2° to 24° for the duration of the experiment. (Sea water in laboratory tanks is from 0° to 1° warmer than where *Tubularia* grows.) One of these, *C*, received air at the same rate as *A*. The other, *D*, received oxygen at the same rate as *B*. Dormant colonies were placed in these containers. After 72 hours there were many hydranths in *B*, less in *A*, and none in *C* and *D*. At 96 hours nearly every free end of the *B* colony had a fully formed hydranth. In *A* they were not so numerous. *D* showed but two small hydranths (occasionally a hydranth is noticed on dormant colonies as they are collected), and *C* showed none.

On August 18, 1938, colonies were collected and placed in flasks as in the previous experiment. The temperature of laboratory ocean water had fallen to 22° C. The experiment was terminated August 24,

TABLE I

See text for explanation.

Flask	Temp. °C.	O ₂ cc./l.	Total hydranths	Hydranths/gm. colony
<i>A</i>	18.4	5.5	243	128
<i>B</i>	18.4	23.5	116	83
<i>C</i>	22.	5.0	0	0
<i>D</i>	22.	21.3	31	38

the colonies removed from the flasks and the number of hydranths carefully counted under the binocular microscope. The oxygen tension in each flask was determined by the Winkler Method. Excess water was removed from the colonies and their weight determined. The results are shown in Table I.

DISCUSSION

Morse (1909) found that *Tubularia* disappeared when the temperature of ocean water rose to 20° C. We observed that in the summer of 1938 it disappeared when the water temperature was 21°. From Morse's experiments and those here reported it seems highly probable

that autotomy of hydranths and the existence of the colonies in a dormant condition is due to high temperature. Conversely, hydranths will be produced in abundance at a few degrees below 20°. North of Cape Cod where the water does not rise above the critical temperature for *Tubularia*, colonies flourish throughout the summer months (Morse, 1909).

This existence of coelenterates in a state of dormancy in regions where conditions during part of the year do not permit active growth has been pointed out by Broch (1925), who called attention to its similarity to spores in plants.

It is doubtful if oxygen in the concentrations normally present in the sea plays a significant rôle in the disappearance or reappearance of active colonies of *Tubularia*. However, in flask D (Table I), kept at 22°, a number of hydranths were formed. The oxygen tension was far above that ever present in the ocean. On bright days the oxygen tension of water bathing the dormant colonies has measured 6.1 cc. per liter, higher than in either flasks A or C (Table I) yet there is no renewed growth as long as the temperature is over 20° C. In the experiment conducted at 24° saturation with oxygen failed to produce a significant number of hydranths. It appears that at temperatures just above the critical point a high oxygen tension may stimulate some hydranth formation but this effect is not noticed at still higher temperatures.

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SUMMARY

When ocean water rises above 20–21° C. in the Woods Hole region the hydranths of *Tubularia crocea* are lost and the coenosarc retreats into the perisarc. The colonies remain in this dormant condition until the temperature drops in autumn. Growth is then resumed.

The dormant colonies can be stimulated to produce hydranths in three days by keeping them at 18.4° C.

It is thought that temperature is the principal agent in the disappearance and reappearance of active colonies.

LITERATURE CITED

- BARTH, L. G., 1937. Oxygen as a controlling factor in the regeneration of *Tubularia*. *Biol. Bull.*, 73: 381.
BARTH, L. G., 1938. Oxygen as a controlling factor in the regeneration of *Tubularia*. *Physiol. Zool.*, 11: 179–186.

- BÄSCHLIN, K., 1932. Histologische Untersuchungen über Rückbildungserscheinungen an Süßwasser- und Meereshydroiden. *Zool. Jahrb., Abt. f. Allg. Zool. u. Physiol.*, **52**: 223-294.
- BROCH, H., 1925. Biogeographie vom Tier aus. *Naturwiss.*, **13**: 447-452.
- CERFONTAINE, P., 1903. Recherches expérimentales sur la régénération et l'hétéromorphose chez *Astroïdes calycularis* et *Pennaria Cavolinii*. *Arch. de Biol.*, **19**: 245-315.
- ELMHIRST, R., 1922. Cyclic conditions and rejuvenation in hydroids. *Nature*, **109**: 208-209.
- GAST, R., AND E. GODLEWSKI, 1903. Die Regulationserscheinungen bei *Pennaria Cavolinii*. *Arch. f. Entw.-mech.*, **16**: 76-116.
- GODLEWSKI, E., 1904. Zur Kenntnis der Regulationsvorgänge bei *Tubularia mesembryanthemum*. *Arch. f. Entw.-mech.*, **18**: 111-160.
- HUXLEY, J. S., AND G. R. DEBEER, 1923. Studies in dedifferentiation. IV. Resorption and differential inhibition in *Obelia* and *Campanularia*. *Quart. Jour. Micr. Sci.*, **67**: 473-495.
- LOEB, J., 1900. On the transformation and regeneration of organs. *Am. Jour. Physiol.*, **4**: 60-68.
- MILLER, J. A., 1937. Some effects of oxygen on polarity in *Tubularia crocea*. *Biol. Bull.*, **73**: 369.
- MORSE, MAX, 1909. The autotomy of the hydranth of *Tubularia*. *Biol. Bull.*, **16**: 172-182.
- RIDDLE, O., 1911. On the cause of autotomy in *Tubularia*. *Biol. Bull.*, **21**: 389-395.
- SUMNER, F. B., R. C. OSBURN AND L. J. COLE, 1913. A biological survey of the waters of Woods Hole and vicinity. *Bull. Bur. Fish.*, **31**: (1911), Part 2: 566.
- THACHER, H. F., 1903. Absorption of the hydranth in hydroid polyps. *Biol. Bull.*, **5**: 297-303.

STANDARDIZATION OF THE PRECIPITIN TECHNIQUE AND ITS APPLICATION TO STUDIES OF RELATIONSHIPS IN MAMMALS, BIRDS AND REPTILES

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Serologic techniques are now used in studies of relationships among bacteria, plants (see review by Chester, 1937) and animals. The types of tests commonly employed are the complement fixation, the agglutination and the precipitation reactions. The particular type of study governs the test employed and there are advantages to each one.

Although it is claimed that the precipitin reaction is not as sensitive as other serologic methods, its simplicity and accuracy make it advantageous in studies of animal relationships where large numbers of tests are necessary. The inconsistency of results, as shown in serologic literature, is possibly not due to the unreliability of the precipitin reaction but rather to the differing techniques of the workers who make the tests. While the techniques that were first employed in the precipitin reaction were important and gave interesting results, they have been much improved in recent years and need still further standardization. There should be a uniform method of measuring the end-point (titer), the chemical and physical factors should be constant and the methods of injection should be standardized.

The precipitin reaction is the one that has been most commonly used in the study of animal kinship, though agglutinins, responsible for blood groups, have been employed in the case of apes and man. Comparatively little research has been done on the serological relationships of reptiles or of birds. Nuttall (1904) stated that there was "a similarity of the blood constitution of all birds." He found differences in degree of reactions but these were so slight that a study like that made with mammalian bloods was not possible. Sasaki (1928) claims that he was able to distinguish the Japanese duck (*Anas domestica erecta*) from the Chinese goose (*Anser cygnoides*) with untreated sera and could distinguish the Japanese duck from the Muscovy duck (*Cairina moschata*) by specialized sera. These results would be more reliable were the possible error of the tests determined. Graham-Smith (1904—see Nuttall, Section VIII) found a closeness of relationship between the Crocodilia and

Chelonia and between the Ophidia and Lacertidia. The reactions indicated that the former groups stood more closely related to the Aves.

Ehrhardt (1929) reviewed the whole field very completely. Some important research has been done recently showing the significance of the tests in determining uncertain relationships. Boyden and Noble (1933) applied the reaction to a study of relationship among several amphibians and clarified the status of these forms. Eisenbrandt's (1938) extension of these methods to chiefly parasitic helminthes is of value in showing that lower animals with only relatively small amounts of circulating proteins may also be successfully studied.

Zuckerman and Sudermann (1935) reported on "Serum relationships within the family Cercopithecidae." As part of their conclusions they state, ". . . their findings support the view . . . that the serum-precipitin test is of limited value in tracing phylogenetic relationships." Their conclusions from their data are not justifiable, however, since these data were obtained by a technique which is questionable.

Magnus (1908) noted that antisera specificity was lessened by continued injections of an antigen. Wolfe (1935, 1936) and Wolfe and Baier (1938) emphasized the importance of the effects of injection methods on the specificity and aspecificity of the serum precipitin tests. It was shown that the type of response is greatly influenced by the method of injection, the specificity being enhanced by limiting the amount of protein injected. With this technique they found greater distinctions among more closely related forms and less "extra-group" reactions. These results suggested that even greater specificity might be possible if the amount of protein injected was reduced to a minimum. It is known that exceedingly small quantities of protein may cause the formation of precipitins in rabbits. Hektoen and Cole (1932) were able to produce antisera of fairly high titers in rabbits by injecting as low as .00029 gram of an ovalbumin solution.

The purpose of this research is: (1) to determine whether precipitin specificity can be increased by limiting the quantity of protein injected into rabbits to a minimum necessary for antibody production; (2) to discover whether a serological relationship study on birds and reptiles is practicable; (3) to attempt to further standardize precipitin technique.

METHODS AND MATERIALS

Healthy adult rabbits of mixed breeds and weighing approximately three kilograms were injected intravenously with serum proteins of ten species of mammals, five species of birds and one species of reptiles. Listed in Table I are the scientific and common names of the animals

TABLE I
Antigens used in experiments

Scientific Name	Common Name	Grams protein per 100 cc. serum
<i>Homo sapiens</i> *	Man	6.843
<i>Pans satyrus</i>	Chimpanzee	6.677
<i>Macacus rhesus</i>	Rhesus macaque	5.425
<i>Macacus sinicus</i>	Bonnet macaque	6.926
<i>Papio hamadryas</i>	Hamadryas baboon	5.530
<i>Lasiopyga mona</i>	Mona guenon	8.616
<i>Cebus capunicus</i>	Capuchin monkey	7.198
<i>Bos taurus</i> *	Ox	6.825
<i>Bison bison</i> *	American buffalo	7.982
<i>Capra hircus</i> *	Domestic goat	6.593
<i>Ovis aries</i>	Domestic sheep	6.051
<i>Odocoileus virginianus</i>	White-tailed deer	5.843
<i>Tamiasciurus hudsonicus</i> *	Red squirrel	6.409
<i>Sciurus carolinensis</i> *	Gray squirrel	8.750
<i>Sciurus niger</i>	Fox squirrel	6.560
<i>Citellus tridecemlineatus</i>	13-lined ground squirrel	5.096
<i>Tamias striatus</i>	Eastern chipmunk	5.139
<i>Marmota monax</i>	Ground hog	6.260
<i>Rattus norvegicus</i>	White rat	7.052
<i>Felis domesticus</i>	Cat	6.825
<i>Canis familiaris</i>	Dog	6.364
<i>Gallus domesticus</i> *	Chicken	4.676
<i>Columba livia</i> *	Pigeon	3.133
<i>Strix varia</i>	Barred owl	4.070
<i>Larus argentatus</i>	Herring gull	3.070
<i>Ardea herodias</i>	Great blue heron	3.550
<i>Mergus merganser</i>	American merganser	3.800
<i>Anser anser</i>	Domestic goose	4.387
<i>Anas boschas</i>	Domestic duck	4.676
<i>Alligator mississippiensis</i>	Alligator	5.158
<i>Phrynosoma cornutum</i>	Texas horned toad	4.389
<i>Chelydra serpentina</i> *	Snapping turtle	3.654
<i>Chrysemys picta</i>	Painted turtle	3.446
<i>Emys blandingii</i>	Blanding turtle	2.299
<i>Clemys insculpta</i>	Wood turtle	3.550
<i>Thamnophis sirtalis</i>	Common garter snake	5.405

* Used for production of antisera and test antigens, others only for test antigens.

from which the blood proteins were secured. The amount of protein per 100 cc. was determined on the whole serum by the Folin-Wright (1919) modified macro-Kjeldahl method. Corrections were not made for non-protein nitrogen. Table II records antisera production data for some of these species. The quantity of protein injected and titers are based on protein calculated from total nitrogen.

The amount of protein injected per animal in the first series has been as low as .000786 gram. This is an exceedingly small amount and not always do such quantities injected into rabbits yield potent antisera. When a second series of injections was given the dosage was usually about one-half the initial series dosage. At present a standardized injection technique is being followed. Rabbits are injected with .001 gram

TABLE II
Antisera production data

Rab- bit No.	Serum protein injected	Injected dil. of solution	Total protein injected	Bled—days after last injection	Titer (dil.)
		<i>cc.</i>	<i>grams</i>		
205	Red squirrel	6.0 (2%)	.00768	12	512,000
215		1.2 (2%)	.001536	12	512,000
136		3.2 (1 : 1)	.102544	14	51,200*
217	Gray squirrel	1.0 (1 : 1000)	.001	10	0
		1.0 (1 : 1000)	.001	12	16,000
		0.75 (1 : 1000)	.0075	8	512,000
223		2.0 (1 : 1000)	.002	10	128,000
125		2.25 (undil.)	.19687	10	51,200*
A11	Ox	1.5 (1 : 500)	.003	2	0
				4	0
				6	16,000
				9	1,024,000
A 9	Buffalo	1 mg./kil.	.0034	10	256,000
A10	Goat	1 mg./kil.	.0034	10	128,000
185	Human	5.75 (2%)	.00786	12	51,200*
77		3.0 (undil.)	.20531	12	102,400*
209		0.6 (2%)	.00082	13	128,000
221		2.0 (1 : 1000)	.002	10	512,000
225	Chicken	2.0 (1 : 1000)	.002	12	0
		0.6 (1 : 500)	.0012	7	512,000
228		0.75 (1 : 1000)	.0015	8	1,024,000
229	Pigeon	1.0 (1 : 500)	.002	10	512,000
222	Snapping turtle	1.0 (1 : 500)	.002	10	0
		0.6 (1 : 500)	.0012	7	256,000

* This notation indicates a serial dilution from a 2 per cent standard—all the rest are from standard solutions containing .001 or .002 gram per cc.

of protein per kilogram body weight distributed over three injections. The injections are made on alternate days. When additional series are necessary the amount administered is one-half that of the initial series given in two injections on alternate days.

Ring tests were used exclusively in determination of the titer. The test consisted of having a duplicate series of serological tubes containing

0.5 cc. of antigen in serial dilution with 0.1 cc. of antiserum layered beneath the antigen. The titer was determined after incubation in a water bath at a temperature of $37.5^{\circ} \pm 1^{\circ} \text{C}$. The possible error in reading the reactions was ± 1 tube. Several experiments consisted of readings at intervals during the sixty minutes in order to study the progression of the titers. Unless definitely specified, the antisera were used undiluted or 1.0 cc. was diluted with 0.25 cc. of a buffered physiological saline solution. This small dilution did not have any effect on the strength of titers in the experiments.

In the earlier experiments the serial dilutions were made from standard test antigens which were 2 per cent solutions. The latter were made by diluting 1.0 cc. of the undiluted blood with 49.0 cc. of buffered saline giving a 1:50 dilution. Since the protein content of the antigens varied

TABLE III
Anti-human sera

Antigen serum proteins of	77	185*	209	221
Man	102,400	51,200	128,000	512,000
Chimpanzee.....	25,600	12,800	128,000	256,000
Rhesus macaque.....	12,800	1,200	0	64,000
Bonnet macaque.....	6,400	1,600	0	64,000
Baboon.....	25,600	400	0	256,000
Mona guenon.....	12,800	400		128,000
Capuchin monkey.....				16,000
Rodentia.....		0		0
Artiodactyla.....	0	0	0	4,000
Carnivora.....	0	0		1,000

* Titers from a 2 per cent solution of undiluted antigen.

from 4 per cent to 9 per cent the titers need adjustment. In the later experiments all serial dilutions are made from standard solutions that contain either .002 or .001 gram protein per cc. thus making all titers comparable.

EXPERIMENTAL DATA

Effects of Amount of Antigen Injected on the Specificity of the Antisera Produced

One of the main purposes of this paper is to show the variance in the degree of the homologous and heterologous precipitin reactions when one factor, the injection procedure, is varied. Antisera 77, 185, 209, 221 are anti-human sera whose reactions are recorded in Table III. Serum No. 77 was produced by injection of .21594 gram protein while the rabbit producing No. 209 received only .00082 gram of protein. (Reference

should be made to Table II for exact detail on quantity of material injected in the experiments to be described.) Of the four antisera 209 is by far the most specific, giving reactions only with chimpanzee and human blood antigens. No. 185 is more specific than 221 even though the latter received one-third the amount of protein given the former.

Injections of the more minute quantities (similar to 185 and 209) usually gave reactions illustrated by these two and more rarely reactions like 221. On the other hand, when 3 cc. of undiluted serum was injected, the most usual response was like that given by No. 77.

Sera 77 and 221 yielded primate group reactions but No. 185 resulted in a sub-grouping showing that the blood proteins of man and chimpanzee are more nearly related to each other than to the blood of the monkeys. Serum 209 was an extremely specific one, giving reactions with only chimpanzee and the homologous antigens. Such antisera would be of great value to the medico-legal workers, as well as to the student dealing with man's closest relatives.

The reactions of three anti-red squirrel and three anti-gray squirrel sera are shown in Table IV. Serum 136 gave a Sciuridae group reac-

TABLE IV
Anti-squirrel sera

Antigen serum proteins of	Anti-red squirrel			Anti-gray squirrel		
	136*	205	215	125*	217	223
Red squirrel.....	51,200	512,000	512,000	2,400	64,000	0
Gray squirrel.....	25,600	0	128,000	51,200	512,000	128,000
Fox squirrel.....	51,200	0	128,000	51,200	256,000	2,000
13-lined gray squirrel...	12,800	0	0	2,400	4,000	0
Chipmunk.....	51,200	0	0	6,400	8,000	0
Ground hog.....	51,200	0	0	3,200		0
Rat.....	0	0		0	0	0
Artiodactyla.....	400		0	200	4,000	0
Primata.....	400			400	4,000	0

* Titers from a 2 per cent solution of undiluted antigen.

tion with no sub-grouping evident, but No. 125, an anti-gray squirrel serum, reacted much more weakly with the blood of red squirrel and other sciurids but could not be differentiated from fox squirrel blood. Both these sera were produced by injection of a much larger quantity of protein than were the other four listed in the table. The latter all show that a definite difference exists between red squirrel blood and that of the fox or gray squirrel bloods. Sera 215 and 217 subdivide the Sciuridae reactions into three sub-groups. Serum 205 is very specific and gave no cross reactions at all and No. 223 allowed for distinction between the

TABLE V
*Anti-aves sera **

Antigen serum protein of	Anti-chicken		Anti-pigeon
	225	228	229
Chicken.....	512,000	1,024,000	0
Pigeon.....	4,000	256,000	512,000
Goose.....	16,000	256,000	0
Duck.....	8,000	256,000	0
Merganser.....	0		
Great blue heron.....	8,000	0	0
Barred owl.....	8,000		
Herring gull.....	0		0
Snapping turtle.....	2,000		0
Painted turtle.....	0	0	0
Alligator.....	0	0	
Common garter snake.....	0	0	0
Cryst. egg albumen of chicken.....	0	0	0

* Fowl and reptile data are from a thesis by Miss Bernice Cohen done under the supervision of the author and used with her permission.

two closely related forms of gray and fox squirrels. This latter result is very rarely secured by the precipitin test with native antisera.

The variation in results that were secured with different dosages do not subtract from the value of the precipitin test but, on the other hand, give it greater possibilities. The differences in reactions seem to imply that a distinction can be made among genera within a family and possibly

TABLE VI
Anti-snapping turtle

<i>Antigen-serum Protein of</i>	<i>No. 222</i>
Snapping turtle.....	256,000
Painted turtle.....	64,000
Blanding turtle.....	16,000
Wood turtle.....	16,000
Alligator.....	0
Horned toad.....	0
Common garter snake.....	0
Aves (7 species).....	0
Cryst. egg albumen of chicken.....	0

species within a genus as well as among larger groups. Similar results have been reported previously but there now seems to be a method whereby these results can be secured consistently. It would be advisable to have many antisera for each group studied in order to insure significant results.

It might be stated that it is believed that the variability of results is not attributable to the innate characteristics of the test but is probably

TABLE VII

Effects of Length of Incubation Period and of Dilution of Antisera

Serum Undiluted					Dilution	
					1 : 3	1 : 5
Incubation Period— Minutes	1	5	10	30	60	60
<i>Anti-buffalo—A9</i>						
Buffalo	0	64,000	128,000	128,000	256,000	512,000
Ox	0	64,000	128,000	128,000	256,000	512,000
Sheep	0	0	0	4,000*	64,000	4,000
Goat	0	0	0	4,000	128,000	4,000
Deer	0	0	0	32,000	128,000	4,000
<i>Anti-goat—A10</i>						
Goat	0	0	0	64,000	128,000	0
Sheep	0	0	0	64,000	64,000	1,000
Buffalo	0	0	0	0	0	0
Ox	0	0	0	0	0	0
Deer					0	
<i>Anti-beef—A11</i>						
Beef	64,000	128,000	512,000	1,024,000	1,024,000	512,000
Buffalo	64,000	128,000	512,000	1,024,000	1,024,000	256,000
Sheep	0	0	0	16,000	128,000	0
Goat					128,000	0
Deer	0	0	0	64,000	256,000	0

* Readings not clear.

due to the antibody producer, in this case, the rabbit. It would be an ideal condition were one able to secure a group of rabbits with similar potentialities for antibody production.

Tests with Avian and Reptilian Blood Sera

There is a meagerness of serological data with respect to reptilian and avian bloods probably due to the conception that the animals within each of these groups are very closely related to each other and are not suitable for study by means of the precipitin reaction. The data listed in Tables V and VI contradict this contention. There are indications that the reactions of anti-avian and anti-reptilian sera produced by using very small amounts of proteins may be useful in confirming present morphological data and thus aiding in the clarification of disputed relationships.

Effects of Incubation Period and Dilution of Antisera on Titers

Previous workers have considered such factors as the length of incubation period and the effect of dilution of the antisera on the homologous and heterologous reactions. The usual method is to make readings after 1 hour incubation at a temperature of $37^{\circ} \pm 1^{\circ}$ C. when the ring test is used and the antisera are used undiluted or diluted to various degrees.

Table VII shows the reactions of three anti-*Artiodactyla* sera which illustrate both time and dilution effects. These antisera readings at 5, 10, and 30 minutes usually showed much greater specificity than did the readings at 60 minutes. Serum A 9 lost its distinguishing characteristic at 60 minutes so that the reactions of buffalo and ox could not be distinguished from deer and goat. It should be noted that antisera A 10 and A 11 show that beef or buffalo can be markedly differentiated from either goat or sheep bloods even at 1-hour readings. Heretofore these great differences have not been shown (with rare exceptions) when undiluted and untreated antisera against the serum proteins were used. By using very small quantities of blood for injections, results similar to, or showing greater differentiation, are consistently secured.

The dilution of antisera with physiological saline is first noted to have an effect on the antigens more distantly related (according to taxonomical schemes). These antisera became much more specific by diluting them as low as 1:3 (2 parts saline and 1 part antiserum). With beef antiserum A11 the reactions of sheep, goat and deer antigens disappeared at antiserum dilutions of 1:3 while the buffalo antigen titer remained similar to the homologous reaction. Such low dilutions show these effects only when the antisera have been produced by the methods described; that is, injections of minute quantities of proteins.

DISCUSSION

Data are presented that show how a greater uniformity of results may be obtained with the serum precipitin reaction. These data further supplement previous work of the author in regard to the specificity and aspecificity of the test. The consistency of the specificity of the reaction with the whole serum proteins of mammalian, avian and reptilian bloods is greater than heretofore reported with untreated rabbit antisera. A distinction of the bloods of such closely related forms as ox and sheep, red squirrel and gray squirrel, several species of turtles and of a number of birds was possible.

The cause of this greater specificity is believed to be the small quantity of protein injected. A standardized quantity is now used; the amount injected in the first series is .001 gram of protein (based on total nitrogen) per kilogram body weight and distributed in three injections. If the titer of the antiserum resulting is insufficient the animal is reinjected with .0005 gram per kilogram body weight given in two injections. This amount injected is thought to be approximately the smallest amount of the antigen used that will regularly cause antibody formation.

The results are not always similar and therefore it seems necessary to produce a number of antisera against a particular antigen in studying relationships and choose those which have the desired specificity. With the present procedures employed the antisera against a particular kind of antigen fall into groups. The reactions of the different groups differ quantitatively but not necessarily qualitatively. Not only are relationships shown but the degree of such relationships can be studied with greater accuracy than before. Morphology does not allow for such a type of approach to the classification of animals. These differences can best be understood by referring to the tables on rodent or primate reactions.

The high specificity secured with a few of the antisera even when used untreated should interest the worker concerned with the medico-legal aspects. Antisera that give reactions only with very closely related bloods should be of much value and no doubt the evidence so secured would be readily accepted by our courts. Such antisera are comparatively easy to produce if the amount of protein injected is reduced to the approximate minimum.

The titer of the reaction at 1 hour incubation did not show the marked differences that were shown after incubating for shorter periods. This factor, the length of incubation period, has not been used with any uniformity for distinguishing closely related proteins. When antisera are produced by the injection of very minute quantities of protein the homologous blood and very closely related ones give ring tests with high titers after 1, 5, 10 or 20 minutes incubation and after that period the blood of more distantly related forms becomes positive. These reactions, when properly performed, are usually very distinct and consistent.

Attempts were made to use readings after incubation of more than 1 hour. These readings are not so clear-cut and not consistent and so are considered unreliable.

The second *in vitro* factor which enhanced the specificity of the reaction, that is, dilution of the antisera, showed a direct correlation with

the results secured by studying the period of incubation. Boor and Hektoen (1930) and Hektoen and Boor (1931), using antihemoglobin sera, found that dilutions with 3 parts of saline or normal rabbit serum eliminated extra specific reactions. Satoh (1933) demonstrated that dilution of 1:10 or less affects the titer of an antiserum when it is produced by few injections with a small quantity of material, and dilution of 1:25 or more affects the reactions when the antisera result from injection of larger quantities given in a greater number of injections. In Satoh's report the dilution seemed to have a similar effect upon the heterologous and homologous reactions. Wolfe (1933) reported that dilutions of 1:2 make the readings of the "ring" clearer but that dilutions of 1:10 do not always eliminate extra-group reactions. These antisera were produced by injection of a relatively large quantity of antigen.

The antisera that were used in the present work were easily affected by small dilutions with buffered saline. The lowest dilution to have an effect was a 1 to 1 (1 part saline) dilution but with some antisera it was necessary to dilute with 2 or 3 parts of saline in order to show decrease in reaction. The dilution always lowered or eliminated the heterologous titer first and one could thus verify the dissimilarities between such closely related bloods as ox and sheep, or man and monkey. But even a higher dilution could not distinguish consistently between ox and buffalo or man and chimpanzee for the dilutions had similar effects upon both titers.

The data presented in this paper are fairly consistent. The methods reported are easily followed and it seems they insure a uniformity of results. The different approaches used resulted in a partial solution of the primary problem concerned; namely, the degree of similarity of the blood proteins of some of the more closely related mammals, birds and reptiles. It seems very possible that a reliable serological relationship study of birds and of reptiles can be accomplished. Additional data may aid in clarifying the "taxonomic confusion" of birds.

Finally—a plea is made to the workers using the serologic method, especially the precipitin technique. It is desirable to obtain uniform and reliable results and one should use the methods that are best adapted to the particular problem. The earlier workers' contribution was and is invaluable but not necessarily the only one that is of use. A modification of the earlier methods is in order and only when one uses the best possible method for the particular study will the results be consistent and therefore biologically significant.

SUMMARY

1. Antibodies were produced in rabbits by injecting very small quantities of serum proteins of mammals, birds and reptiles.
2. Antisera produced by injection of a minimum quantity of antigen were the most specific as determined by the "ring" method.
3. Occasionally the serum proteins of such closely related animals as ox and sheep or gray squirrel and red squirrel could be distinguished.
4. Antiserum dilutions as low as 1:3 at times eliminated the heterologous reaction.
5. The "ring" appeared in the homologous and very closely related protein solutions earlier than in the more distantly related ones when incubated at temperatures of $37^{\circ} \pm 1^{\circ} \text{C}$.
6. It was not possible to distinguish between the buffalo and ox serum proteins or between the goat and sheep serum proteins.
7. Serological relationship studies of birds and reptiles seem feasible.
8. The procedures have not been entirely new but the necessity for uniformity and standardization of methods has been emphasized.

LITERATURE CITED

- BOOR, ALDEN K., AND LUDVIG HEKTOEN, 1930. Preparation and antigenic properties of carbonmonoxide hemoglobin. *Jour. Inf. Dis.*, **46**: 1.
- BOYDEN, ALAN, AND G. K. NOBLE, 1933. The relationships of some common Amphibia as determined by serological study. *Am. Mus. Novitates*, No. 606: 1.
- CHESTER, K. STARR, 1937. A critique of plant serology. *Quart. Rev. Biol.*, **12**: 19-46; 165-190; 294-321.
- EHRHARDT, ALBERT, 1929. Die Verwandtschaftbestimmungen mittels der Immunitätsreaktionen in der Zoologie und ihr Wert für phylogenetische Untersuchungen. *Ergebn. u. Fortschr. der Zool.*, **7**: 279.
- EISENBRANDT, LESLIE L., 1938. On the serological relationship of some Helminths. *Am. Jour. Hyg.*, **27**: 117.
- FOLIN, O., AND L. E. WRIGHT, 1919. A simplified macro-Kjeldahl method for urine. *Jour. Biol. Chem.*, **38**: 461.
- HEKTOEN, LUDVIG, AND ALDEN K. BOOR, 1931. The specificity of hemoglobin precipitins. *Jour. Inf. Dis.*, **49**: 29.
- HEKTOEN, LUDVIG, AND ARTHUR G. COLE, 1932. Precipitinogenic action of minute quantities of ovalbumin. *Jour. Inf. Dis.*, **50**: 171.
- MAGNUS, WERNER, 1908. Weitere Ergebnisse der Serum-Diagnostik für die theoretische und angewandte Botanik. *Ber. deut. Bot. Gesell.*, 1908, **26a**: 532.
- NUTTALL, GEORGE H. F., 1904. Blood Immunity and Blood Relationship. Cambridge University Press.
- SASAKI, KIYOTSUNA, 1928. Serological examination of the blood relationships between wild and domestic ducks. *Jour. Dept. Agr. Kyushu Imperial Univ.*, **2**: 117.
- SATOH, TAKEO, 1933. Ueber Präzipitintiter und "Präzipitingehalt." *Zeitschr. f. Immunitätsforsch. u. exp. Therapie*, **79**: 117.
- WOLFE, HAROLD R., 1933. Factors which may modify precipitin tests in their application to Zoölogy and Medicine. *Physiol. Zool.*, **6**: 55.

- WOLFE, HAROLD R., 1935. The effect of injection methods on the species specificity of serum precipitins. *Jour. of Immun.*, **29**: 1.
- WOLFE, HAROLD R., 1936. The specificity of precipitins for serum. *Ibid.*, **31**: 103.
- WOLFE, HAROLD R., AND JOSEPH G. BAIER, JR., 1938. Comparison of quantitative precipitin techniques as influenced by injection procedures. *Physiol. Zool.*, **11**: 63.
- ZUCKERMAN, S., AND ANN E. SUDERMANN, 1935. Serum relationships within the family Cercopithecidae. *Jour. Exp. Biol.*, **12**: 222.

EXPERIMENTS ON LIGIA IN BERMUDA

VI. REACTIONS TO COMMON CATIONS

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Five preceding papers of this series (Barnes, 1932, 1934, 1935, 1936, 1938) have described the effect of salts and other factors on the littoral isopod *Ligia baudiniana*. The present report deals with the negative reaction of *Ligia* to filter paper moistened with salt solutions and the survival in sea water containing a higher percentage of K, Ca, or Na.

Reaction to Filter Paper Moistened with Salt Solutions

Large filter papers (diameter 25 cm.) were cut in two, each half saturated with a different solution and placed in a covered flat dish in a photographic dark room having a light of 61.9 foot candles directly above the center of the dish. Five isopods (freshly collected) were released in the dish and their distribution on the two halves was observed at five to fifteen-minute intervals for a period of one to two hours. The dish was rotated 90° after each observation to eliminate any unsuspected source of orientation. With distilled water on both sides the distribution was approximately equal.

It is known (Barnes, 1938) that the isopods tend to collect on the distilled water side when the other half of the paper is moistened with sea water and the present experiments were designed to test the salts separately. The aversion for sea water is shown by specimens previously kept in air on seaweed moistened with sea water (Barnes, 1938) and by specimens washed rapidly in distilled water (Barnes, 1935), but it was found that prolonged immersion in distilled water destroys this reaction. Thus in the present experiments of 168 specimens previously immersed separately in 100 cc. of distilled water for an average of half an hour, 82 collected on the sea water side and 86 on the distilled water paper.

For $\frac{5}{8}$ M NaCl vs. distilled water, the ratio is the same as for sea water vs. distilled water, and in both cases the negative reaction no longer occurs at 50 per cent dilution (Table I). The aversion for KCl paper

was still evident in a dilution of 10 per cent (see Table I). The isopods showed a positive reaction to CaCl_2 paper compared with distilled water paper and an "all-or-none" reaction to CaCl_2 occurred when the other half contained NaCl. An even distribution occurred between CaCl_2 and LiCl paper and the negative reaction to LiCl compared with distilled water was less than that to the other salts.

Survival in Modified Sea Water

The toxic action of Na, Ca and K was studied by increasing the proportion of each salt in sea water. Specimens were tested separately in 100 cc. of mixtures of sea water and isotonic salt solution (Table II).

TABLE I

Reaction of Ligia to filter paper saturated with salt solutions.
(The animals were tested in groups of five.)

Treatment of each half of paper	Total number of isopods on each half	Ratio
5/8 M NaCl vs. distilled water	71 : 127	1 : 1.78
75 per cent 5/8 M NaCl vs. distilled water	85 : 120	1 : 1.41
50 per cent 5/8 M NaCl vs. distilled water	73 : 77	1 : 1.05
25 per cent 5/8 M NaCl vs. distilled water	83 : 72	1 : 0.86
5/8 M NaCl vs. $\frac{3.5}{8}$ M CaCl_2	1 : 63	1 : 63.00
100 per cent 5/8 M KCl vs. distilled water	37 : 147	1 : 3.97
75 per cent 5/8 M KCl vs. distilled water	26 : 82	1 : 3.15
50 per cent 5/8 M KCl vs. distilled water	31 : 81	1 : 2.61
25 per cent 5/8 M KCl vs. distilled water	56 : 82	1 : 1.46
10 per cent 5/8 M KCl vs. distilled water	61 : 99	1 : 1.62
5 per cent 5/8 M KCl vs. distilled water	96 : 104	1 : 1.08
$\frac{3.5}{8}$ M CaCl_2 vs. distilled water	105 : 55	1 : 0.52
$\frac{3.5}{8}$ M CaCl_2 vs. 5/8 M LiCl	22 : 22	1 : 1.00
5/8 M LiCl vs. distilled water	49 : 63	1 : 1.28

The isopods showed a high tolerance for sea water containing excessive sodium or calcium, but increasing the KCl content is rapidly fatal. The toxicity of Na is a smooth function of the concentration. The Ca curve is erratic and the K curve falls abruptly for sea water mixtures containing over $\frac{1}{16}$ M KCl (see Fig. 2).

Survival in Oxygenated Solutions

The wide variation in survival times in a given salt solution suggests

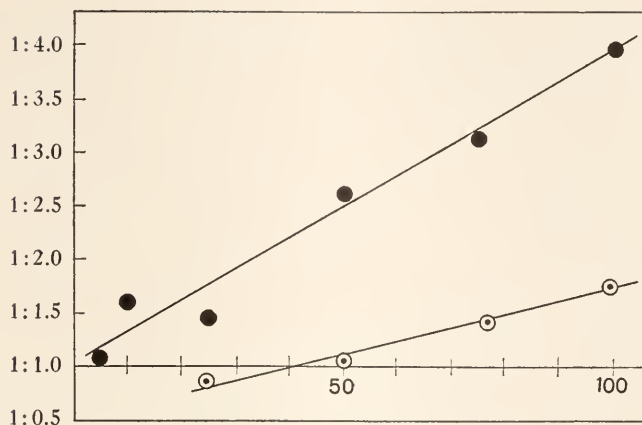


FIG. 1. Negative reaction to filter paper containing salt solutions. Ordinates: ratio of the number of isopods on the salt solution side to the number on the distilled water side. Abscissae: percentage concentration in cc. of 5/8 M salt. Solid circles: KCl. Open circles: NaCl (see Table I).

that other factors besides the direct chemical effect of the ions may be involved. To eliminate effects of asphyxiation oxygen was bubbled

TABLE II

Survival of Ligia in modified sea water

Parts of salt solution in 100 cc. of modified sea water	Average survival	Maximum survival	Number of specimens
	<i>hours</i>	<i>hours</i>	
9 per cent 5/8 M NaCl.	69.8±15.2	136	9
50 per cent 5/8 M NaCl.	59.4±4.5	168	39
71.4 per cent 5/8 M NaCl.	30.9±3.5	79	20
80 per cent 5/8 M NaCl.	20.7±0.8	24	37
90 per cent 5/8 M NaCl.	8.0±0.03	11	43
10 per cent 5/8 M KCl.	44.7±5.6	112	19
11 per cent 5/8 M KCl.	36.5±5.5	48	8
20 per cent 5/8 M KCl.	10.2±0.7	17	19
30 per cent 5/8 M KCl.	3.7±0.4	6	8
40 per cent 5/8 M KCl.	5±0	5	5
50 per cent 5/8 M KCl.	0.7±.08	1	10
30 per cent $\frac{3.5}{8}$ M CaCl ₂	68.5±10.8	264	25
50 per cent $\frac{3.5}{8}$ M CaCl ₂	34.2±5.7	242	24
70 per cent $\frac{3.5}{8}$ M CaCl ₂	39.1±6.7	254	19
90 per cent $\frac{3.5}{8}$ M CaCl ₂	38.8±2.9	62	17

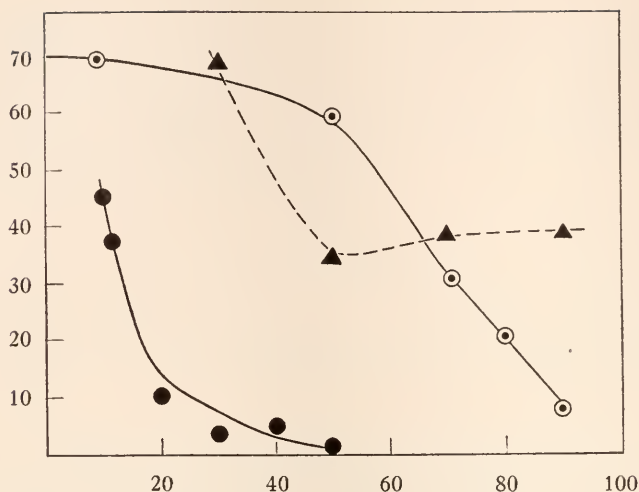


FIG. 2. Survival times of isopods in sea water in which the concentration of one ion is increased. Ordinates: average survival in hours. Abscissae: concentration of $5/8$ M KCl and NaCl and of $3.5/8$ M CaCl_2 in cc. per 100 cc. of modified sea water. Solid circles: KCl; open circles: NaCl; triangles: CaCl_2 .

through the 100 cc. of salt solution in several cases, but did not affect the survival times in distilled water, $5/8$ M NaCl or $5/8$ M KCl (Table III).

TABLE III

Survival in oxygenated solutions

Medium	Average survival	Maximum survival	Number of specimens
	<i>hours</i>	<i>hours</i>	
Distilled water.....	4.0 ± 0.2	6	8
$5/8$ M NaCl.....	8.7 ± 0.6	11	4
$5/8$ M KCl.....	1.6 ± 0.1	3	7

Discussion

It is of interest to note that the aversion for sea water is similar to aversion for NaCl and in both cases the negative reaction ceases at half dilution. The quantity of KCl in sea water is below the threshold for the negative reaction to this salt, while CaCl_2 has no repellent action when compared with distilled water.

It was suggested (Barnes, 1938) that the salts on the paper stimulate the isopods to greater movement which compels them to collect on the salt-free side. The pronounced aversion for KCl and positive reac-

tion to Ca support this hypothesis. The rather precise relation between the concentration of NaCl and KCl and the magnitude of the response may also be cited. It is unfortunate that our quantitative knowledge of chemical stimulation is very meager compared with the extensive studies of stimulation by light and gravity. The ratio of effect of K *vs.* distilled water to Na *vs.* distilled water is 2.2, somewhat greater than the ratio of ionic mobilities, i.e., 1.5. Hopkins (1932) has shown the importance of ionic mobility in stimulation by salts. The reaction of the animals when each side of the substratum is treated with a different salt is not an additive effect of the reactions to each salt compared with distilled water paper. Thus the ratio for CaCl_2 *vs.* distilled water is 1:0.52 and NaCl *vs.* water is 1:1.78 but the ratio for CaCl_2 *vs.* NaCl is as high as 63.0:1. This striking difference probably results from the combined action of the inhibiting Ca and stimulating Na on the leg movements. The differences cannot be explained by toxicity alone. Thus Li, the most toxic of the ions, gives a 1:1 ratio with Ca.

In the case of sea water *vs.* distilled water the salt requirements of the animals must also be considered. Thus if depleted of salt by a half hour's immersion in distilled water the negative reaction to sea water disappears. The rôle of the flushing mechanism for the gills by which water rises between the last pair of legs is not known. Several animals were observed with the legs in position for the capillary conduit, but there was not sufficient solution on the filter paper to flush the gills.

The specific effect of Na, Ca, and K is indicated in the toxicity curves for sea water containing increasing concentrations of each ion. The smooth relation between concentration and toxicity for Na suggests a gradual modification of a normal process in the animal. The very steep KCl curve indicates severe toxic action and the lack of correlation with concentration in the case of calcium is characteristic of a surface reaction. It was shown (Barnes, 1938) that the protective action of Ca in hypotonic sea water also resembles a surface reaction.

The experiments in which oxygen was introduced into the solutions help to show that the reported survivals of immersed animals are true salt effects. This is especially important in the case of K which paralyzes all gill movement. It is probable that oxygen would increase the survival in less toxic solutions in which the animals live for several days.

Summary

1. The negative reaction of *Ligia* to filter paper moistened with $\frac{5}{8}$ M NaCl is similar to the negative reaction to sea water. In both cases dilution by 50 per cent destroys the effect.

2. The aversion for filter paper containing KCl and NaCl is approximately a rectilinear function of the concentration.

3. The results can be explained by the assumption that the ions stimulate the legs and the greater activity results in a distribution on the salt-free side of the substratum.

4. When presented with a "choice" between filter paper containing CaCl_2 and NaCl, there is an "all-or-none" aversion for the NaCl.

5. The survival of *Ligia* in sea water containing increasing concentrations of Na, K and Ca is described.

6. The survival in distilled water or in solutions of single salts is not affected by oxygenation.

The writer is indebted to Dr. J. F. G. Wheeler for many courtesies.

LITERATURE CITED

- BARNES, T. C., 1932. Salt requirements and space orientation of the littoral isopod *Ligia* in Bermuda. *Biol. Bull.*, **63**: 496.
- BARNES, T. C., 1934. Further observations on the salt requirements of *Ligia* in Bermuda. *Biol. Bull.*, **66**: 124.
- BARNES, T. C., 1935. Salt requirements and orientation of *Ligia* in Bermuda III. *Biol. Bull.*, **69**: 259.
- BARNES, T. C., 1936. Experiments on *Ligia* in Bermuda. IV. The effects of heavy water and temperature. *Biol. Bull.*, **70**: 109.
- BARNES, T. C., 1938. Experiments on *Ligia* in Bermuda. V. Further effects of salts and of heavy sea water. *Biol. Bull.*, **74**: 108.
- HOPKINS, A. E., 1932. Chemical stimulation by salts in the oyster, *Ostrea virginica*. *Jour. Exper. Zool.*, **61**: 13.

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SOME POLYCLADS OF THE NEW ENGLAND COAST,
ESPECIALLY OF THE WOODS HOLE REGION

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This report is based on polyclads collected at Woods Hole, Massachusetts, Mt. Desert Island, Maine, Connecticut and Long Island, New York. I believe it includes practically all of the species expected to occur in the neighborhood of Woods Hole. The Woods Hole specimens were personally collected in the summer of 1935 or were loaned for identification by Mr. George Gray, Curator of the Museum of the Marine Biological Laboratory. The Peabody Museum of Yale University kindly loaned some specimens collected by Verrill along Massachusetts and Connecticut shores. The specimens from Mt. Desert Island, Maine, were personally collected in the summer of 1937. The specimens from Connecticut and Long Island were presented by Dr. Herman Spieth and Mr. M. D. Burkenroad.

Ten species are represented in the material; none of these is new but most of them are interesting as old species badly in need of reworking. Each species has been carefully studied whole and in serial section to determine its status according to modern taxonomic standards. It is gratifying to me to be able to clear up the taxonomic position of some of the old species of Girard and Verrill.

The scheme of classification here adopted is taken from Bock's invaluable treatise (1913) which is fundamental to all modern work on the group.

Before proceeding to the taxonomic descriptions, it seems desirable to give a brief account of the reproductive system of polyclads, since this system is of paramount importance in many genera. Polyclads are hermaphroditic with numerous testicular and ovarian follicles scattered throughout the parenchyma; there are no yolk glands. Fine vasa efferentia connect the testes to the paired vasa deferentia, expanded, thin-walled, usually sinuous canals, packed with sperm. These separately or after a union enter the male copulatory apparatus, which typically consists of seminal vesicle, prostate or granule vesicle, and penis. The seminal vesicle is a rounded, oval or curved sac with a thick muscular

wall. In some cases (*Stylochus* in part, some species of *Notoplana*) the terminal parts of the vasa deferentia acquire muscular walls and entirely resemble the seminal vesicle, so that a tripartite seminal vesicle results (Fig. 1). Certain glands known as granule (German workers) or prostate (English workers) glands are constantly associated with the male apparatus of polyclads. They may open directly into the terminal part of the male canal but more often are associated into a definite rounded or oval body with a muscular wall, termed the granule or prostate vesicle. The granule glands are often included inside this vesicle or may open into it from the exterior (extracapsular glands of Bock) or both. The granule vesicle may form a direct part of the male canal so that the sperm must pass through its lumen as in the Leptoplanidae (Fig. 5); this condition of the vesicle will here be termed "interpolated" ("eingeschaltet" in German). In some acotylean families and in the Cotylea in general the granule vesicle is "free," that is, connects by a duct to the male canal (Fig. 1). The function of the granule glands is unknown but the secretion is ejaculated with the sperm and must be of importance in their physiology. After passing through or receiving separately the granule vesicle, the male duct enters the penis proper, typically having the form of a projecting conical muscular papilla. It is frequently provided with a long or short hard stylet, sometimes curved or coiled, and may then itself be much reduced. The penis papilla may project directly into the male atrium or antrum or it may be at the inner end of a long canal leading from the atrium; this canal is termed by Bock the penis pocket (Fig. 5). When a penis pocket is present there may be at the place where the pocket widens into the male atrium, a penis-like projection, the penis sheath. In the family Planoceridae there is no proper penis but instead a cirrus, armed with teeth or spines. Penis here is limited to a male copulatory organ which is simply protruded by muscular action; cirrus means a copulatory organ which is everted when used so that the lining is turned to the outside.

The ripe eggs commonly collect in paired thin-walled expanded canals, the uteri, which in the family Leptoplanidae unite in front of the pharynx. The uteri, separately or after a union, open into the common unpaired female canal which then proceeds to the genital pore. This canal from the genital pore to the entrance of the uteri is commonly muscular and receives innumerable cement glands; Bock terms it vagina since it must serve for copulation in most polyclads. It is also the passage by which the eggs reach the exterior. Near the genital pore it may be widened into a cement pouch into which most or all of the cement glands open. The cement glands secrete the material by which the eggs are fastened into ribbons or masses and stuck to objects. In many poly-

clads the female canal continues inward proximal to the entrance of the uteri and terminates in a small or large spherical, oval, or tubular sac, now known as Lang's vesicle (Fig. 5). This appears to be a seminal receptacle since sperm are often seen in it.

The male apparatus is always anterior to the female apparatus. The genital pores are commonly separate but may be united. Paired or multiple male apparatuses are not uncommon.

SUBORDER ACOTYLEA LANG 1884

Definition.—Polyclads without a sucker behind the female genital pore; pharynx ruffled; copulatory apparatuses behind the middle of the body; tentacles when present of the nuchal type; uteri anterior to the female genital pore.

SECTION CRASPEDOMMATA BOCK 1913

Definition.—Acotylea with marginal eyes, in addition to the usual cerebral and tentacular eyes.

FAMILY STYLOCHIDAE STIMPSON 1857 (EMEND. BOCK, 1913)

Definition.—Craspedommata with a band of eyes along the whole or part of the body margin; female or both genital pores very near the posterior end; granule vesicle free; tentacles generally present; body usually oval, thick, firm and opaque.

GENUS STYLOCHUS EHRENBURG 1831 (EMEND. LANG 1884)

Definition.—Stylochidae with male and female pores close together in the posterior fourth of the body; tentacles well-developed; pharynx large with well-marked folds; prostate vesicle large, chambered, with extracapsular gland cells; seminal vesicle single or tripartite; Lang's vesicle absent.

STYLOCHUS ZEBRA (VERRILL) 1882

Syn. *Stylochopsis zebra* Verrill 1882.

Stylochus zebra (Verrill) 1892.

Description.—The external features of this familiar Woods Hole polyclad have been adequately described by Verrill (1892). The body is of elongated oval or oblong form, obtuse at each end, of thick and firm texture, reaching a length of 30–40 mm. The dorsal surface has a color pattern of alternating yellowish or white and brown or chocolate cross-bars of which the most anterior and posterior ones are V-shaped. Near the anterior end are two rounded tentacles filled with eyes and between the tentacles are the paired cerebral groups of eyes. These

are loose elongated groups extending behind and before the level of the tentacles. The margin is completely encircled with a band of small eyes, wider in front, diminishing caudally.

Study of sections of the copulatory apparatus proves for the first time that this species was correctly placed by Verrill in the genus *Stylochus*. The uteri and vasa deferentia course alongside the posterior part of the very large, much ruffled pharynx behind the posterior end of which occurs the copulatory apparatus. The rear ends of the vasa deferentia expand and acquire muscular walls as they ascend to join the seminal vesicle proper, producing the three-parted type of seminal vesicle characteristic of several species of *Stylochus* (Fig. 1). The central lobe of this or seminal vesicle proper continues posteriorly as the ejaculatory duct which narrows and becomes less muscular, receiving near the penis the duct of the prostate vesicle. The latter is a large oval chambered sac with thick muscular walls penetrated by the necks of extracapsular granule glands (Fig. 1). The erect position of the prostate vesicle is unusual for the genus; the vesicle is commonly horizontal. After receiving the prostatic duct the ejaculatory duct continues through the penis, a simple conical projection into the small male atrium. The latter opens below by the male genital pore. Shortly behind this occurs the female genital pore, from which the muscular ciliated vagina extends dorsally, then curves posteriorly and downward to terminate where it receives the uteri (Fig. 1). A Lang's vesicle is absent as in the genus in general.

The color pattern and the erect prostatic vesicle serve to distinguish this species from others of the genus.

Distribution.—Region of Woods Hole, on wharves and pilings and along shores; also found by Verrill at other places in Vineyard Sound and in Long Island Sound, near New Haven, Conn., in dead shells of *Sycotypus* occupied by hermit crabs.

STYLOCHUS ELLIPTICUS (GIRARD) 1850, NEW COMB.

Syn. *Planocera elliptica* Girard 1850.

Stylochopsis littoralis Verrill 1873.

Stylochus littoralis (Verrill) Lang 1884.

Eustylochus ellipticus (Girard) Verrill 1892.

I am fortunately able to give a description of this species and establish its correct systematic position. There are available two vials of Verrill's collecting, a specimen in the Woods Hole material, and a number of individuals presented by Dr. Spieth and Mr. Burkenroad whom I here wish to thank for their trouble. Study of this material proves that

the animal is a typical member of the genus *Stylochus* and hence that Verrill was in error in erecting a new genus *Eustylochus* for the species (Verrill, 1892). *Eustylochus* thus becomes a synonym of *Stylochus*.

Description.—*S. ellipticus* (Fig. 2) is of oval or elliptical form and may reach a length of 20–25 mm.; specimens as small as 4 mm. were found to have the male system fully developed but the female system was immature. The body is flat but somewhat thick and opaque with undulated margins. The color is stated by Verrill and others to be various shades of cream, yellow, or brown, veined or reticulated with a lighter shade, or freckled with golden brown on a lighter ground color; the periphery is clear and translucent and there is usually a light cream to brown stripe in the posterior middorsal region. There are two nuchal tentacles filled with eyes; these are probably elongated and pointed in life but in the preserved specimens were rounded and somewhat sunk into depressions. Between and in front of the tentacles occur the cerebral eyes, subject to much variation. Not infrequently these occur in two pairs of groups of two eyes each (Fig. 2), but often there are three to five or more (up to nine were seen) eyes in each of the four groups and there may be a few scattered eyes farther back over the brain. Specimens have also been seen in which the cerebral eyes were not definitely arranged in four groups but formed a loose band on each side. A band of eyes occurs along the margin of the anterior third to half of the body; these eyes are larger in the mid-anterior region and diminish in size and number around the sides of the body. In many specimens no eyes could be seen along the margin of the posterior half or two-thirds of the body but in some individuals there are a few small eyes scattered along this region so that the entire margin in such cases is encircled with eyes.

The pharynx is of moderate size, smaller proportionately than in *Stylochus zebra* but well folded. The branches of the digestive tract radiate to the periphery making a reticulum through numerous anastomoses between adjacent branches.

Study of the copulatory apparatus in serial sections (Fig. 3) shows that the species is a typical *Stylochus* and that no grounds exist for separating it into a distinct genus. The genital pores are very close together near the posterior margin. The coiled vasa deferentia run alongside the pharynx and their distal ends turn dorsally and become muscular as they join the seminal vesicle proper. There is thus some development of the tripartite condition of the seminal vesicle seen in several species of *Stylochus*, but the central lobe is here much larger than the lateral lobes formed by the ends of the vasa deferentia. The central lobe tapers posteriorly into the ejaculatory duct which runs beneath the

prostatic vesicle to open into the penis lumen. The prostatic vesicle is a very large oval sac with a chambered interior and a thick muscular wall penetrated by the necks of extracapsular granule glands which discharge into the chambers of the interior. The prostatic vesicle has the horizontal orientation characteristic of the genus. Its duct joins the ejaculatory duct in the interior of the penis and the common duct so formed continues to the penis tip (Fig. 3). The penis papilla is a moderately muscular conical projection armed with a short stylet situated in the lumen. The occurrence of a stylet appears to be unusual in the genus *Stylochus*. The expanded male atrium closely surrounds the penis papilla and exits below by the male genital pore. The lining of the male atrium and the base of the penis papilla are filled with glandular secretion.

The large female pore, situated immediately behind the male pore, is lined by a very tall epithelium also having a glandular border of eosinophilous secretion. The vagina accompanied by cement glands proceeds dorsally and forward as a narrow tube which near the dorsal body wall turns sharply backwards and terminates by receiving the uteri.

Distribution.—Stated by Verrill to occur from New Haven, Conn., to Casco Bay, Maine, under stones in shallow water and tide-pools; not uncommon now at Cold Spring Harbor, Long Island, and along the shores of the west end of Long Island Sound where it joins the East River. Apparently scarce along the Connecticut shore, where four specimens were taken under a rock at Indian Neck, by M. D. Burkenroad. One specimen lent by Mr. Gray came from the bottom of a light-ship, off Woods Hole. Verrill's specimens from the Peabody Museum were as follows: one vial containing six animals, labelled Savin Rock (this is

For all figures the numbers listed below have the same connotations.

1, marginal eyes, 2, tentacles, 3, tentacular eyes, 4, cerebral eyes, 5, brain, 6, pharynx, 7, vasa deferentia, 8, enlarged muscular terminations of the vasa deferentia, 9, seminal vesicle, 10, prostate vesicle, 11, glandular chambers of the prostate vesicle, 12, extracapsular granule glands, 13, penis papilla, 14, ejaculatory duct, 15, prostatic duct, 16, stylet, 17, male genital pore, 18, male atrium, 19, penis pocket, 20, female genital pore, 21, vagina, 22, cement pouch, 23, cement glands, 24, entrance of the uteri into the vagina, 25, uteri, 26, stalk of Lang's vesicle, 27, Lang's vesicle, 28, cirrus, 29, retractor muscle of the cirrus, 30, protractor muscle of the cirrus, 31, musculo-glandular fold of the vagina, 32, main intestine, 33, penis sheath, 34, female genital atrium, 35, granule glands, 36, common genital pore, 37, pouch from genital atrium.

PLATE I

FIG. 1. Copulatory apparatus of *Stylochus zebra*, sagittal view.

FIG. 2. *Stylochus ellipticus*, dorsal view of whole mount.

FIG. 3. *Stylochus ellipticus*, sagittal view of copulatory apparatus.

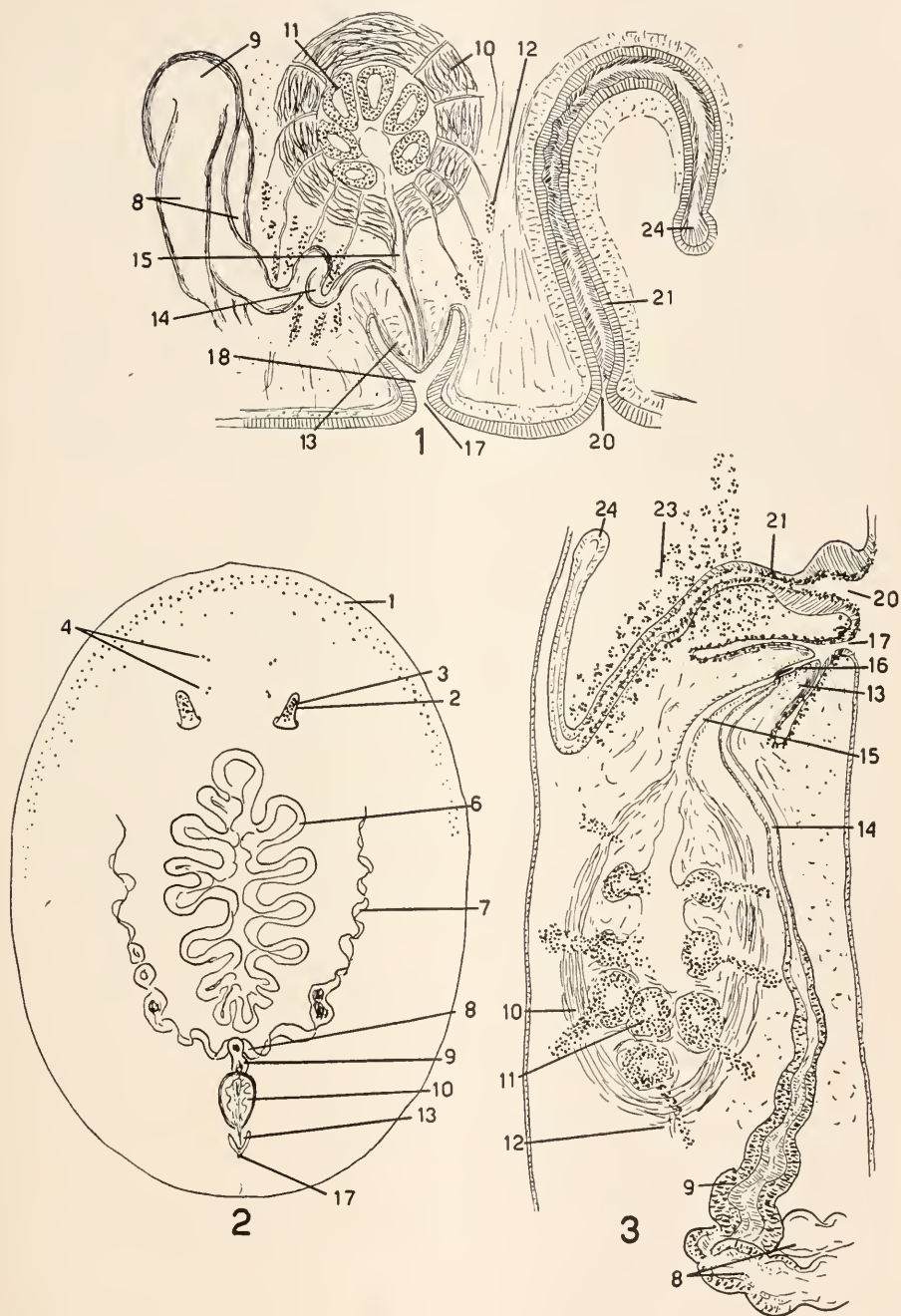


PLATE I

near New Haven), under stones, Oct. 1871; and one vial containing one animal labelled *Stylochus littoralis*, New Haven, Oct. 1872.

SECTION SCHEMATOMMATA BOCK 1913

Definition.—Acotylea without marginal eyes; commonly with paired cerebral and tentacular clusters of eyes close together and far back from the anterior margin.

FAMILY LEPTOPLANIDAE STIMPSON 1857 (EMEND. BOCK)

Definition.—Schematommata with flat thin elongated bodies; prostatic vesicle, when present, interpolated; male apparatus directed backwards; definite tentacles usually absent but may be present; dorsal surface colored, generally some shade of brown; uteri confluent in front of the pharynx.

GENUS NOTOPLANA LAIDLAW 1903

Syn. *Leptoplana* (in part).

Definition.—Leptoplanidae with true seminal vesicle and chambered prostatic vesicle into which the ejaculatory duct penetrates; male apparatus close behind the pharynx, far removed from the posterior margin; penis with or without a stylet; eyes in four clusters; tentacles generally absent or rudimentary.

It has been necessary to limit the genus *Leptoplana* into which it was formerly customary to throw almost any leptoplanid species to the first recognizable species ascribed to *Leptoplana*, namely *L. tremellaris* (O. F. Müller) 1774, and to other species which can be shown to be similar to this species. It unfortunately happens that the anatomy of *L. tremellaris* is somewhat aberrant and hence very few species remain in the genus *Leptoplana*. I know of none on the Atlantic coast of the United States but one occurs in Puget Sound and some may exist on the California coast although most of the Pacific coast species called *Leptoplana* must be transferred to other genera. With this limitation of the genus *Leptoplana*, the majority of the typical leptoplanids fall into the genus *Notoplana*, a genus of many species, all so much alike externally that identification is practically impossible without recourse to serial sections of the copulatory complex. Only one species of *Notoplana* is so far known from the Atlantic coast of North America but many occur on the Pacific coast.

NOTOPLANA ATOMATA (O. F. MÜLLER) 1776

Syn.? *Leptoplana variabilis* (Girard) Verrill 1892.

Description.—This species has already been well described by Bock (1913) and hence I shall be brief. The species, seen at Mt. Desert Island, Maine, may reach a length of 28 mm. and has the typical external aspect of the genus. The elongated oblong body is broadest across the anterior fourth and then tapers to the rounded posterior end (Fig. 4). Young specimens are even more tapering than fully grown ones. The color is grayish brown above, more or less flecked and streaky. There are the usual four clusters of eyes, two rounded tentacular clusters representing the sites of the aborted tentacles and consisting of 6–10 large eyes and a few small ones; and the elongated cerebral clusters extending forward from the brain and composed of about 15–40 eyes of various sizes, but smaller than the largest of the tentacular eyes. The number of eyes increases with age so that young specimens have few eyes.

The whole anatomy is typically leptoplanid with a central elongated ruffled pharynx, radiating anastomosed digestive branches, uteri encircling the pharyngeal pocket, and vasa deferentia looping backwards across the stalk of Lang's vesicle (Fig. 5). The details of the copulatory apparatus have been discussed by Bock (1913) who presents a sagittal view and I give a ventral view (Fig. 5) made from a pressed live specimen. The vasa deferentia enter separately the angles of the rounded muscular seminal vesicle from which the ejaculatory duct penetrates more than halfway into the lumen of the spherical thick-walled chambered prostatic vesicle. From this the elongated curved penis pocket leads to the male atrium. A very small penis papilla next the prostatic vesicle bears the moderately long curved stylet which occupies the penis pocket and often protrudes from the male genital pore. There is a penis sheath. The wide muscular vagina continues beyond the entrance of the uteri forward, then bends backwards as a narrow stalk terminating in an enlarged Lang's vesicle (Fig. 5).

Bock is of the opinion that *Leptoplana variabilis* is identical with *Notoplana atomata*. While the former is undoubtedly a species of *Notoplana*, I find some points in Verrill's description of it (1892) which do not fit my observations on *N. atomata*. Thus Verrill gives the shape of *L. variabilis* as oblong or elliptical while *N. atomata* is always wedge-shaped, often markedly so, and describes the penis stylet as much longer and more coiled than I have seen it. I have not seen any other shape in a number of specimens examined than the simple bend figured.

Locality.—*Notoplana atomata* appears to be widely distributed along shores of the North Atlantic coast from Scandinavia to Maine; under stones, Mt. Desert Island, Maine, and probably northward.

GENUS EUPLANA GIRARD 1893

Syn. *Discoplana* Bock 1913.

Definition.—Leptoplanidae of somewhat elongated form, without a prostatic vesicle; genital pores close together; tentacles lacking; penis unarmed, mostly small or even absent; Lang's vesicle present or absent. Type: *Euplana gracilis* (Girard) 1850.

I regret the necessity of making Bock's genus *Discoplana* a synonym of *Euplana* but the circumstances leave me no other course.

EUPLANA GRACILIS (GIRARD) 1850

Syn. *Prosthiostomum gracile* Girard 1850.

Prosthiostomum gracile Girard, Verrill 1892.

Euplana gracilis Girard 1893.

A number of specimens of a polyclad considered unnamed by members of the invertebrate staff at Woods Hole were secured in 1935. Study of serial sections revealed that the animal belonged to Bock's genus *Discoplana* and for some time the form was regarded as a new species of *Discoplana*. Then Verrill's figure of *Prosthiostomum gracile* Girard was recognized as identical with the polyclad in question (Verrill, 1892, p. 497). The question then arose whether the species actually is Girard's species, as Verrill thought. It is my opinion that there is very little doubt of this, although Girard was entirely mistaken in placing the animal in *Prosthiostomum*. Later, 1893, Girard recognized this error and created for the species the new genus *Euplana*. This genus appears to be quite valid under the rules and therefore I must regretfully declare *Discoplana* Bock 1913 a synonym of *Euplana* Girard 1893. The correct name of the animal is then *Euplana gracilis* (Girard).¹

PLATE II

FIG. 4. *Notoplana atomata*, from life, dorsal view, Mt. Desert Island, Maine.

FIG. 5. Dorsal view of copulatory apparatus of *Notoplana atomata* as seen in live pressed specimen.

FIG. 6. *Euplana gracilis*, from life, dorsal view, Woods Hole, Mass.

FIG. 7. Another view of the anterior end of *Euplana gracilis*, from life.

FIG. 8. Sagittal section of the copulatory apparatus of *Euplana gracilis*.

¹ If the identity of the species under consideration with *Prosthiostomum gracile* be questioned, then *Discoplana* would again become a valid genus and the animal would be a new species of *Discoplana*.

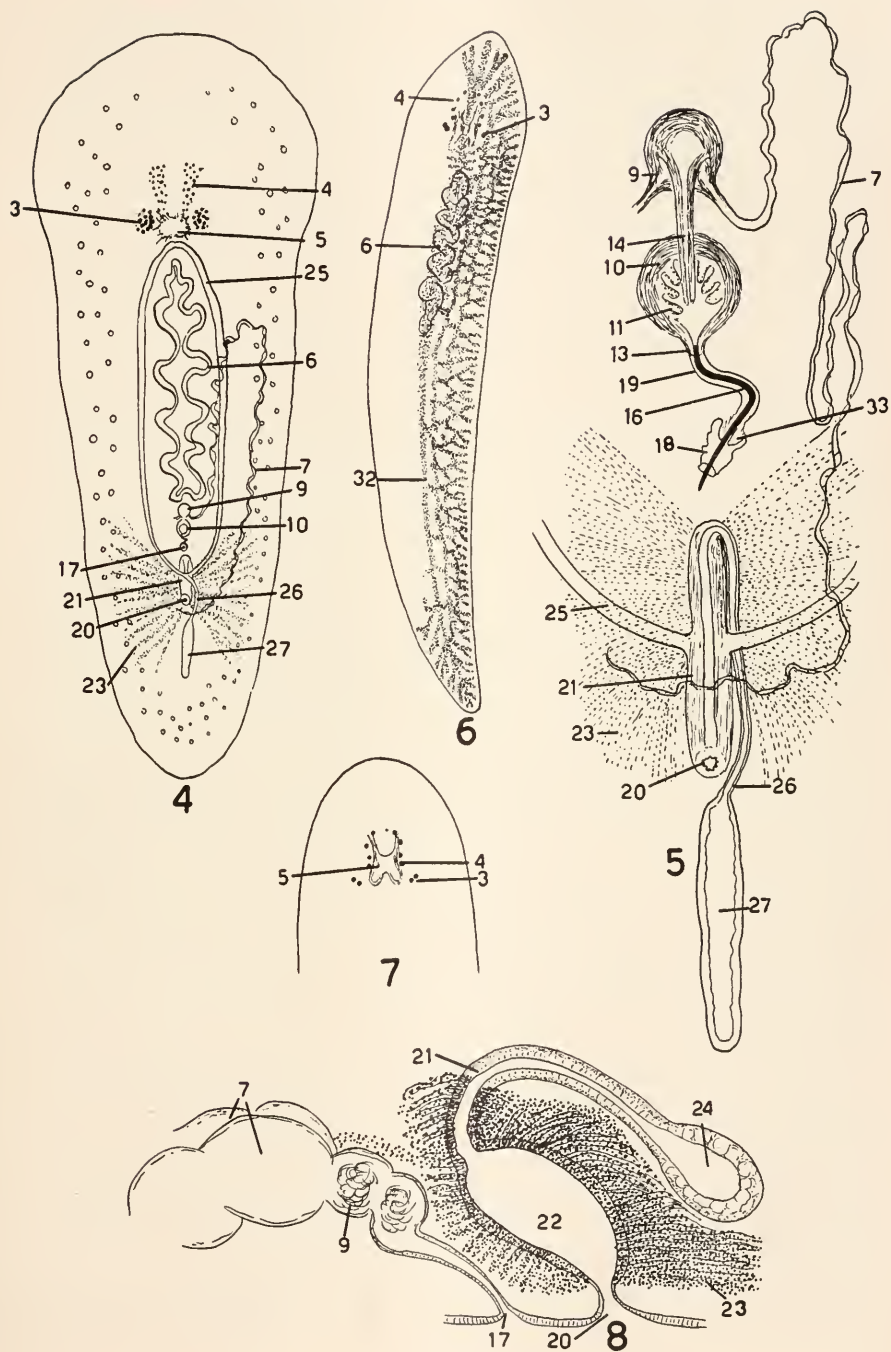


PLATE II

Description.—*E. gracilis* is of slender elongated shape, resembling a fresh-water planarian (Fig. 6). The anterior end is at times somewhat pointed (Fig. 6), at other times rounded (Fig. 7). From the level of the brain the body tapers gradually to the obtuse or rounded posterior end. The species is small, with a maximum length of 8 mm. and of yellowish gray or brownish gray coloration. The eyes are the most distinctive external feature and readily serve to distinguish this species from all other North American polyclads. There are typically six eyes on each side, four in a lengthwise row, representing the cerebral clusters, and two eyes obliquely placed close together, representing the tentacular clusters. Specimens have been seen with some slight irregularity of eye number, such as one or two additional eyes, but the described arrangement is the rule. There are no marginal ocelli as supposed by Verrill.

The small, slightly ruffled pharynx is situated far forward, a position explaining the mistake in placing the species in *Prosthlostomum*. Behind the pharynx the median intestinal trunk is conspicuous in whole mounts (Fig. 6). From this trunk lateral branches extend to the periphery.

The main feature of the copulatory apparatus (Fig. 8) is the absence of the prostatic vesicle. The male and female genital pores are situated close together some distance behind the pharynx at about the middle of the body. Behind the pharynx the expanded thin-walled vasa deferentia unite to a but slightly muscular seminal vesicle which tapers directly to the male genital pore (Fig. 8). There is no penis papilla and no prostate vesicle nor are any extracapsular granule glands discernible in the available material. The female pore shortly behind the male pore leads into an expanded cement pouch into which open great numbers of cement glands (Fig. 8). The vagina then curves posteriorly and ventrally and terminates where it receives the two uteri. There is no Lang's vesicle. The uteri are confluent in front of the pharynx, then course along the sides of the pharynx behind which they expand considerably before joining the vagina.

E. gracilis closely resembles in its sexual anatomy several other species of *Euplana* (= *Discoplana*) differing chiefly in the total absence of a penis papilla.

Distribution.—Found in abundance in the Eel Pond at Woods Hole, Mass., on wharves and pilings among masses of hydroids and sponges; apparently formerly common in Boston Harbor and along the Connecticut shore.

Neotype.—One whole mount; paratype, one set of sagittal sections, deposited in the museum at Woods Hole.

GENUS *STYLOCHOPLANA* STIMPSON 1857 (EMEND. BOCK)

Definition.—Leptoplanidae with true seminal vesicle and non-chambered prostate vesicle, the latter not penetrated by the ejaculatory duct; tentacles present or absent; eyes in paired cerebral and tentacular groups; separate or fused genital pores, which may be near the posterior margin.

In general aspect the species of this genus closely resemble those belonging to *Notoplana*. The essential difference between them lies in the prostate vesicle which is not chambered and not penetrated by the ejaculatory duct.

STYLOCHOPLANA ANGUSTA (VERRILL) 1892

Syn. *Leptoplana angusta* Verrill.

[not *Stylochoplana angusta* (Verrill) Palombi 1928].

The finding at the Peabody Museum of a vial containing three specimens which are undoubtedly "*Leptoplana*" *angusta* has enabled me to determine the systematic status of this species. It was found to be a leptoplanid which fits best into the genus *Stylochoplana* although not entirely conforming to the present conception of this genus. It seems preferable, however, to expand the definition of the genus than to create a new genus for the species.

In 1928 Palombi identified a *Stylochoplana* species obtained by the Suez Canal expedition as *Leptoplana angusta*, and called it *Stylochoplana angusta* (Verrill). This identification is erroneous as the species in question differs decidedly from the latter. Professor Palombi has been informed of the necessity of creating a new specific name for his species.

Description.—*S. angusta* is stated by Verrill to be of elongated elliptical form, thin, with flexible undulated margins, rounded anterior end, and notched posterior margin. Figure 9 is a drawing of a whole mount made from one of Verrill's specimens; the other two were sectioned sagittally. The maximum length is given by Verrill as 12–16 mm., the breadth 4–6 mm., and the color as various shades of light brown. The cerebral and tentacular eyes form a continuous band on each side (Fig. 9), of which the posterior group of larger eyes on either side of the brain undoubtedly represents the tentacular eyes while the band of smaller eyes extending forward from these are the cerebral eyes. The narrow ruffled pharynx is somewhat posterior in position and this presumably accounts for the very posterior location of the copulatory complexes.

There is a large common genital pore close to the posterior margin shortly in front of the notch. This continues into a genital atrium which curves anteriorly receiving the vagina in its dorsal wall and the penis in its anterior wall. The small male copulatory complex (Fig. 10) conforms to that of the genus in general. The vasa deferentia after a union enter the anterior end of the elongated oval seminal vesicle. From this the curved ejaculatory duct runs to the prostatic vesicle. This is of oval form with a thin muscular wall and the interior lined by granule glands. No extracapsular granule glands were seen in the sections; naturally after sixty years in preservative the animals are not in very good histological condition. From the prostate vesicle, the ejaculatory duct continues backward and enters the small pyriform penis which projects into the male portion of the atrium as a slight conical eminence. The very long vagina runs from the roof of the atrium far forward and then after a short backward bend opens into a large spherical Lang's vesicle (Fig. 10). The uteri join the backward bend so that the stalk of Lang's vesicle is very short. The vagina is slightly muscular along its entire course and is accompanied throughout by cement glands. I am unable to determine from my material whether the uteri are confluent in front of the pharynx but Verrill's statements indicate that this is the case. In the whole mount, the uteri are much distended with eggs (Fig. 9) and extend anteriorly beyond the brain, a condition unusual in the Leptoplanidae.

The two sets of sagittal serial sections are identical except for one very puzzling feature. In one of them, the larger of the two, the common genital atrium continues dorsally behind the vaginal entrance as a wide pouch (Fig. 10), which appears to open on the dorsal surface, presumably into the posterior notch. The specimen is unfortunately imperfect at this place so that I cannot be sure of the actual presence of an opening there; but all appearances indicate that this is the case and I have so drawn the region in question (Fig. 10). The second specimen (Fig. 11) shows no trace whatever of such a pouch although it appears to be quite mature sexually. More material will be necessary to settle the matter of the occurrence and relations of the pouch in question. It may represent a ductus vaginalis, as in Bock's genus *Ceratoplana* (Bock, 1925).

Sty. angusta differs from the typical members of the genus in the far posterior location of the genital pore and the forward course of the vagina. The first point is not of so much importance since Bock (1924) found a Japanese species which he assigned to *Stylochoplana* and which also has a very posterior common genital pore. Kato (1937) reports

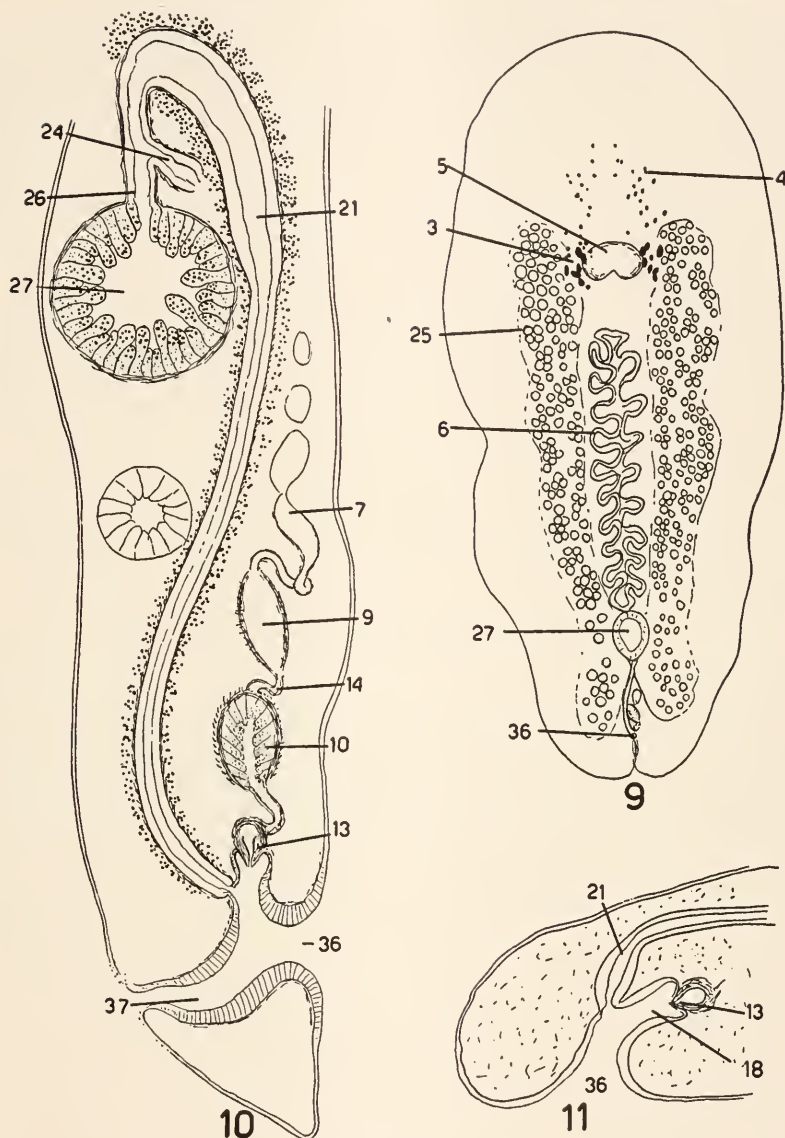


PLATE III

FIG. 9. *Stylochoplana angusta*, dorsal view of whole mount, neotype.

FIG. 10. Sagittal view of the copulatory apparatus of *Stylochoplana angusta*, from one of the sets of serial sections, showing problematical pouch from genital atrium dorsally.

FIG. 11. Terminal part of the copulatory apparatus of *Stylochoplana angusta*, from the second set of serial sections, showing absence of a pouch.

another Japanese *Stylochoplana* having the same feature. But in these species, the vagina, despite the posterior situation of the genital pore, makes the usual backward bend, so that Lang's vesicle lies well behind the genital pore. The situation found in *Sty. angusta* with the long anterior extension of the vagina and the location of Lang's vesicle much anterior to the male apparatus appears to be unique. One could consider it a basis for the erection of a new genus but I prefer to leave the species in *Stylochoplana*, at least for the present.

Distribution.—*Stylochoplana angusta* was found by Verrill at Provincetown, Mass., 1879, on the bottom of a whaling vessel recently arrived from the Carolina coast. The polyclad probably came from this region, especially as it was associated with other animals said by Verrill to be southern. The vial from the Peabody Museum contained a label on which was printed: Off Cape Cod, U. S. F. C. 1879, and written: *Leptoplana*, vessel bottom.

Neotype.—Whole mount designated as neotype; one set of sagittal serial sections, designated as paratype; both deposited in the Peabody Museum, Yale University.

GENUS HOPLOPLANA LAIDLAW 1902

Syn. *Planocera*, Group B, Lang 1884.

Definition.—Schematommata without a true seminal vesicle or penis papilla; ends of the vasa deferentia enormously enlarged into thick-walled muscular tubes serving as accessory seminal vesicles; penis stylet fastened directly to the prostate vesicle; tentacles present; with cerebral and tentacular eye clusters, the latter closely embracing or in the tentacle bases; no Lang's vesicle; shape oval or rounded.

In 1902 Laidlaw rightly removed some of Lang's species of *Planocera* to a new genus *Hoploplana*, which does not even belong to the family Planoceridae. It also does not entirely fit into the family Leptoplanidae, so that Bock (1913) left it as an appendix to the latter. It would seem to me desirable to place *Hoploplana* in a family by itself despite the fact that its affinity to the Leptoplanidae is indicated by the finding in Puget Sound of a species of *Notoplana*, *N. segnis* Freeman 1933, which has also very enlarged, highly muscular terminations of the vasa deferentia.

HOPLOPLANA GRUBEI (GRAFF) 1892

Syn. *Planocera grubei* Graff 1892.

This species, common on the Sargassum, has been adequately described and figured by von Graff (1892). Three specimens, brought

to me for identification by Dr. Hadley of the Invertebrate staff at Woods Hole, were taken from the Sargassum in Vineyard Sound, August 3, 1938. The coloration was stated to consist of gray reticulations on a pinkish-brown or flesh ground. M. D. Burkenroad of the Peabody Museum, who has collected a number of specimens of this species from the Sargassum in the Gulf of Mexico and North Atlantic, reported the color as consisting of livid white reticulations on a brown ground. It is clear that von Graff's conception of the color, based on preserved material, is erroneous. Preserved specimens give no idea of the original coloration. A colored figure of this species made by Dr. J. F. G. Wheeler of the Bermuda Biological Station is being published in my article on the polyclads of Bermuda and the Sargassum (Hyman, 1939).

HOPLOPLANA INQUILINA (WHEELER) 1894

Syn. *Planocera inquilina* Wheeler 1894.

Hoploplana inquilina (Wheeler) Bock 1913.

This species, familiar at Woods Hole, was rightly removed by Bock to the genus *Hoploplana*. Wheeler presents an excellent figure of the entire animal but his account of the internal details leaves much to be desired. I have therefore deemed it necessary to make a study of serial sections.

Description.—The expanded thin-walled vasa deferentia packed with sperm course alongside the pharynx near the posterior end of which they alter into thick-walled muscular accessory seminal vesicles (Fig. 12). These, after reaching a level behind the male pore narrow, approach the median line, and fuse to a narrow duct, the common vas deferens. This runs anteriorly penetrating into a round somewhat muscular organ which Wheeler considers the penis. It is, in fact, the prostatic vesicle and this mistake accounts for Wheeler's inability to find such a vesicle. It is not, to be sure, a very typical prostate. It has an excessively thick wall, containing muscle fibers coursing parallel to the surface contour and in the center a small area of granule glands radiating around the central small ejaculatory duct. The granule glands are best seen in frontal section (Fig. 12) as they are oriented parallel to such a section. Scattered glands also seem to extend into the thick wall of the prostate vesicle. After passing through the glandular region the ejaculatory duct terminates in the base of the stylet which constitutes the entire penis (Fig. 13). It is directed ventrally in the available specimens, extending into the male atrium. The latter has quite a muscular wall and is lined by an extremely tall columnar epithelium which bulges from the genital pore (Fig. 13).

It will be seen from the foregoing description that the male apparatus of *H. inquilina* accords completely with that found in other members of the genus. The vasa deferentia form accessory seminal vesicles, there is no true seminal vesicle, and the prostate vesicle bears directly the penis stylet without the intervention of any penis papilla proper.

The female copulatory apparatus consists simply of the vagina which extends dorsally from the genital pore receiving the secretion of the thickly strewn cement glands. The vagina is somewhat muscular, and lined by tall slender epithelial cells. After making a backward bend, the vagina constricts and beyond this constriction the shell glands cease and the lining epithelium alters to large vacuolated cells. This portion of the vagina resembles histologically a Lang's vesicle but morphologically is not such since the uteri open into its ventral end (Fig. 13).

Distribution.—*Hoploplana inquilina* has been recorded only from the vicinity of Woods Hole where it inhabits the mantle cavity of the large gastropod *Sycotypus*.

FAMILY PLANOCERIDAE Lang 1884 (EMEND. BOCK 1913)

Definition.—Schematommata with a cirrus instead of a penis; cirrus lined with spines, hooks, or ridges eversible to the exterior; with tentacles; with tentacular and cerebral eyes; copulatory complexes immediately behind the pharynx; with true or accessory seminal vesicles; prostate vesicle free or interpolated.

GENUS GNESIOCEROS DIESING 1861²

Syn. *Pelagoplana* Bock 1913.

Definition.—Planoceridae with wedge-shaped pellucid bodies; with tentacles containing eyes; cerebral eyes also present; with a true seminal vesicle and interpolated prostatic vesicle; cirrus armed with parallel toothed bands; vagina with a powerful musculo-glandular fold; Lang's vesicle transverse. Type, *Gnesioceros sargassicola* (Mertens) 1833.

PLATE IV

FIG. 12. Frontal view of the vasa deferentia and prostate vesicle of *Hoploplana inquilina*, showing area of granule glands in the center of the vesicle around the ejaculatory duct.

FIG. 13. Sagittal view of the copulatory apparatus of *Hoploplana inquilina*.

FIG. 14. *Gnesioceros verrilli*, dorsal view, whole mount, Woods Hole specimen.

² In 1861 Diesing created the genus *Gnesioceros* for two species; "*Planaria*" *pellucida* and "*Planaria*" *sargassicola* both described by Mertens in 1833. *Planaria pellucida* belongs to the genus *Planocera* and was removed to that genus in 1844 by Oersted. Diesing did not indicate which of the two species he considered typical of the genus *Gnesioceros*. I hereby remove *pellucida* from the genus and declare *sargassicola* to be the type of *Gnesioceros*, thus making the genus valid.

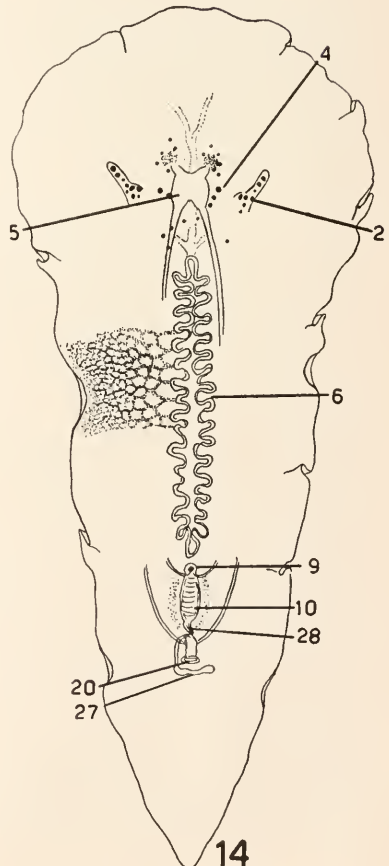
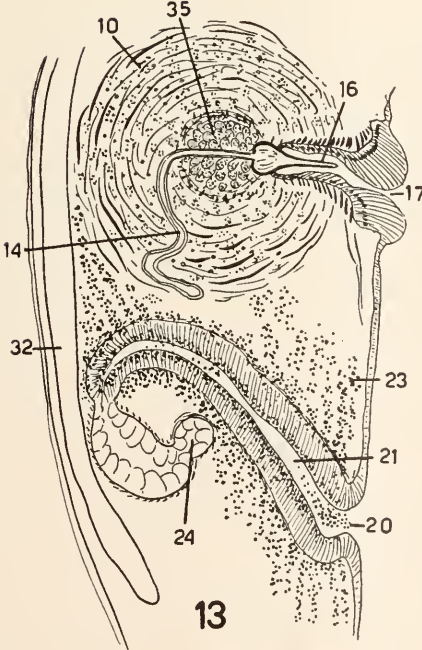
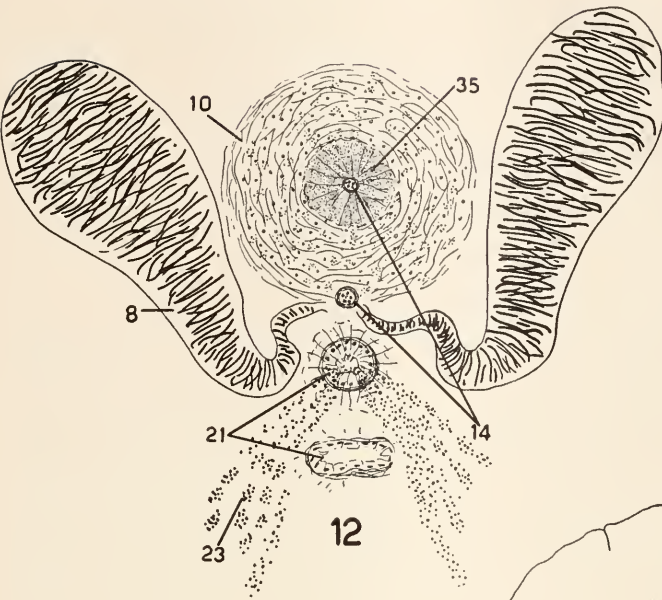


PLATE IV

GNESIOCEROS SARGASSICOLA (MERTENS) 1833

Syn. *Planaria sargassicola* Mertens 1833.

Stylochus sargassicola Ehrenberg 1836.

Planocera sargassicola Oersted 1844.

Stylochus pelagicus Moseley 1877.

Gnesioceros mertensi Diesing 1861.

Stylochus sargassicola (Mertens) Lang 1884.

Stylochoplana sargassicola (Mertens) Graff 1892.

Pelagoplana sargassicola (Mertens) Bock 1913.

This is the common polyclad of the Sargassum found on the floating weed in various parts of the world, common in the Gulf of Mexico, Caribbean, and North Atlantic. I took several specimens alive from the Sargassum at Bermuda and Burkenroad collected hundreds of specimens during an expedition to study the 'biology of Sargassum inhabitants. Alive the animal is pellucid with small brown spots and an expanded anterior end tapering to a pointed posterior end. It has been figured and described by von Graff (1892) and I have given an account of the structure of the cirrus (Hyman, 1939). A specimen of this species was taken from the Sargassum in Vineyard Sound, August 3, 1938.

GNESIOCEROS VERRILLI, NEW NAME

Syn. *Imogine oculifera* (Girard) Verrill 1892.

(not *Imogine oculifera* Girard 1853).

In 1882 Verrill found in Quisset Harbor a single specimen of a polyclad which he assigned to Girard's species *Imogine oculifera*. This identification is clearly erroneous since Girard plainly states that *Imogine oculifera* has a band of eyes completely encircling the margin and hence is a stylochid while Verrill's figure plainly shows a planocerid. Two vials of specimens were obtained from Mr. Gray which I am confident are identical with Verrill's specimen. Examination of these animals proved that they belong in the genus *Gnesioceros*. As it is necessary to give a specific name to Verrill's species, I propose to call it *verrilli*.

Description.—*G. verrilli* is very similar in all respects to *G. sargassicola*. The graceful body is anteriorly expanded (Fig. 14), being widest at about the level of the brain, and from there tapers gradually to the pointed posterior tip. The species is small, around 6-8 mm. in length. The preserved specimens were colorless but Verrill gives the color as carmine red. As this color was stated, however, to be limited to the intestinal branches, it is nearly certain that it resulted from

ingested food and that the animal is in fact, like *G. sargassicola*, pellucid with small brownish marginal spots. There are two elongate nuchal tentacles, each containing several eyes, most of which are in the tentacle bases. Between the tentacles, extending some distance behind and in front of them, are two loose elongate groups of cerebral eyes, of about 8–12 eyes each (Fig. 14). From the long, narrow, slightly ruffled pharynx a rich net of anastomosing intestinal branches radiates to the periphery.

The copulatory complexes behind the pharynx are very similar to those of *G. sargassicola* which have been described by Graff, 1892, and Hyman, 1939. The vasa deferentia enter separately the ventral angles of the pyriform seminal vesicle (Fig. 15) which ascends and narrows to the ejaculatory duct opening into the anterior end of the prostate vesicle. The latter is an elongate structure divided into chambers by cross-partitions and covered with a thick muscular coat which operates the cirrus. The prostate vesicle continues directly into the cirrus, a curved beak-like structure composed of hard yellow material, presumably not chitin but an albuminoid. The structure of the cirrus was described and figured in another place (Hyman, 1939) and hence a brief statement will be made here. The cirrus possesses a median groove and thus resembles a conch shell. It is made of parallel toothed bands (Fig. 16) which can be pulled into the interior along the groove by the action of the retractor muscle fastened along one side of the prostate vesicle. The protractor muscle on the other side of the prostate vesicle is attached to the central part of the bands. When it contracts, it pulls the bands to the outside where they take a horizontal course. The cirrus is situated in a pocket which continues into the male atrium and so to the male genital pore.

The female genital pore is shortly behind the male pore. From it the ciliated, slightly muscular vagina extends dorsally, then curves backward and ventrally and receives the uteri. Beyond this union the female canal continues as the stalk of Lang's vesicle and opens into the latter. Lang's vesicle is unique in the genus *Gnesioceros* in taking a transverse course, so that it embraces the rear and sides of the female genital pore in a somewhat crescentic shape. The genus is further notable for the presence in the vagina of a powerful musculo-glandular fold, consisting of a heavy muscle mass to which are attached numerous gland cells. In *G. sargassicola* this fold is ring-shaped, completely encircling the vagina; but in *G. verrilli* it is well-developed only in the anterior wall of the vagina, the side facing the cirrus, and but slightly present and more diffuse in the posterior vaginal wall (Fig. 15). The epithelium over the main muscle mass is reduced to a cuticularized membrane. In *G. sar-*

gassicola the vaginal epithelium from the musculo-glandular fold to the genital pore is also cuticularized and thrown into lengthwise ridges; but this is not the case in *G. verrilli*.

These differences in the vaginal structure constitute the chief distinctions between *G. sargassicola* and *G. verrilli*. Other differences are: the pharynx appears to be narrower and less ruffled in *G. verrilli* than in *G. sargassicola* and the prostate vesicle narrower and longer relative to the cirrus region in the former than in the latter.

Neotype.—It has seemed desirable to designate a neotype in the form of a whole mount deposited in the museum at Woods Hole; and a set of serial sections is also presented to this institution.

Distribution.—Taken from the eel grass, Devil's Foot Island, Woods Hole, Mass., December, 1930 and October 28, 1931; also found by Verrill in Quisset Harbor, Buzzard's Bay, on sandy bottom in four or five fathoms, September 4, 1882.

SUBORDER COTYLEA LANG 1884

Definition.—Polyclads with a sucker behind the female genital pore (a few exceptions); pharynx ruffled to tubular; copulatory complexes generally in the anterior half of the body; prostatic vesicle, when present, always free; uteri generally behind the female genital pore; tentacles when present of the marginal type; eyes on the anterior margin, on the tentacle bases when these are present.

FAMILY EURYLEPTIDAE LANG 1884

Definition.—Cotylea with anteriorly directed tubular pharynx; male apparatus below or immediately behind the pharynx, directed forward, with a free prostate vesicle and armed with a stylet; with or without uterine glands; tentacles vary from well-developed pointed marginal folds to none; with paired cerebral clusters of eyes and paired marginal clusters which are on the tentacles when these are present.

GENUS EURYLEPTA EHRENBURG 1831 (EMEND. LANG 1884)

Definition.—Euryleptidae with well-developed pointed tentacles; main intestine with very few (up to about five) lateral branches; male

PLATE V

FIG. 15. Sagittal view of the copulatory apparatus of *Gnesioceros verrilli*.

FIG. 16. Prostate vesicle and cirrus of *Gnesioceros verrilli*.

FIG. 17. Tentacles and eye distribution of *Eurylepta maculosa*, specimen collected by Verrill.

FIG. 18. Sagittal section of the same specimen as Fig. 17, showing pharynx and female copulatory apparatus; because of damage the male apparatus, to be expected immediately behind the pharynx, is missing.

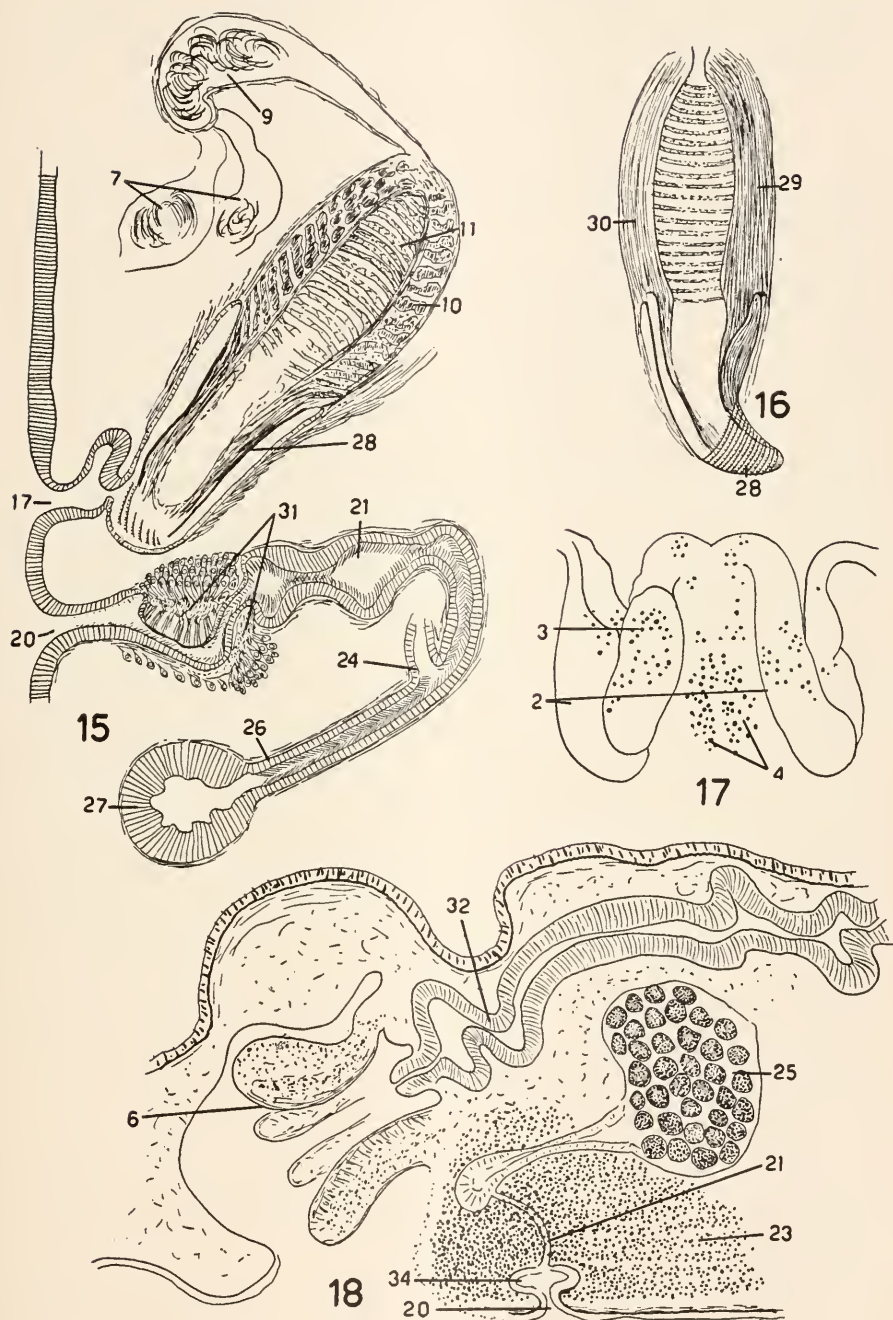


PLATE V

apparatus beneath the posterior end of the pharynx; uterine glands mostly one pair or none.

EURYLEPTA MACULOSA VERRILL 1892

A single specimen of this species was found at the Peabody Museum; after the eye arrangement had been drawn (Fig. 17) the animal was cut into sagittal sections but these were not very satisfactory. However, it seems desirable to describe the findings.

Description.—The species is said by Verrill to have a thin, changeable, elliptical or oblong body, 10–12 mm. long, with thin often undulated margin. The anterior margin is upfolded to make two fairly long bluntly pointed tentacles which bear eyes on their anterior faces (Fig. 17). There are, as also noted by Verrill, no eyes at or near the tentacle tips. There are elongated paired cerebral groups of eyes which are continued forward to the margin between the tentacles by scattered clusters of eyes. The preserved specimen was distinctly mottled with brown and white; Verrill gives the color as pale, yellowish or pinkish white, irregularly specked and mottled or veined with purplish or brown.

The serial sections show a typical euryleptid structure (Fig. 18) with short tubular pharynx directed forward. From the pharynx the main intestinal trunk runs backward in the middorsal line and has about three pairs of wide lateral branches, thus conforming to the genus *Eurylepta*. To either side of the intestinal trunk is a large thin-walled uterus stuffed with eggs. No uterine glands have been found. The region immediately behind the pharynx where the male copulatory apparatus would be expected is unfortunately broken and no trace of the male copulatory apparatus has been found. The female apparatus (Fig. 18) appears to be completely present. The female pore, shortly behind the pharynx, leads into a small expanded atrium from which a narrow vagina, receiving the innumerable cement glands, proceeds dorsally. The great mass of the cement glands almost obscures the female duct. The vagina widens into a small chamber from which a duct runs posteriorly and receives the uteri.

There appears to be little doubt that the species was correctly placed in the genus *Eurylepta*.

Distribution.—Found by Verrill on piles, in mud, and among algae at low tide at Woods Hole and vicinity, stated to be uncommon. The Peabody Museum specimen was labelled: *Eurylepta maculosa* V. cotype, mud, Woods Hole, Aug. 2nd.

Specimen.—The set of sections made from the specimen has been returned to the Peabody Museum.

Whole mounts or preserved specimens of most of the species described in this paper (except those belonging to the Peabody Museum) have been deposited in the Museum at Woods Hole. In some cases a set of serial sections has also been presented to this institution.

SUMMARY

1. *Stylochus zebra* (Verrill) 1882 is shown to have been correctly placed by Verrill in the genus *Stylochus*.

2. *Eustylochus ellipticus* (Girard) 1850 is shown to be a typical member of the genus *Stylochus*, its name thus becomes *Stylochus ellipticus*, and *Eustylochus* becomes a synonym of *Stylochus*.

3. *Notoplana atomata*, usually erroneously called *Leptoplana variabilis*, is the most common polyclad of the Maine coast.

4. *Prosthlostomum gracile* Girard 1850 has been rediscovered at Woods Hole. The later name *Euplana gracilis* which Girard gave to this species is valid and *Discoplana* Bock 1913 becomes a synonym of *Euplana* Girard 1893.

5. Specimens of *Leptoplana angusta* Verrill 1892 have been found at the Peabody Museum, Yale University. The species is placed in the genus *Stylochoplana* although it differs somewhat from the typical members of the genus. The name is then *Stylochoplana angusta* (Verrill).

6. As noted by Bock (1913), *Planocera inquilina* Wheeler 1894 must be transferred to the genus *Hoploplana* and the correct name of the species is *Hoploplana inquilina*. What Wheeler considered to be the penis is in reality the prostatic vesicle.

7. Two common and well-known Sargassum polyclads have been taken on the Sargassum in Vineyard Sound; these are *Hoploplana grubci* (Graff) 1892 and *Gnesioceros sargassicola* (Mertens) 1833. *Pelagoplana* Bock 1913 becomes a synonym of *Gnesioceros* Diesing 1861.

8. Another species of *Gnesioceros*, *G. verrilli*, new name, occurs around Woods Hole; it was seen by Verrill in 1882 and erroneously identified by him as *Imogine oculifera*.

9. A single specimen of *Eurylepta maculosa* Verrill 1892 was found at the Peabody Museum; some details of its anatomy are described.

ADDENDUM

Since this manuscript was sent to press there have appeared three articles on Atlantic coast polyclads by A. S. Pearse and associates (*Proc. U. S. Nat. Mus.*, 86, *Jour. Elisha Mitchell Sci. Soc.*, 54, and *Bull. Mt. Desert Is. Biol. Lab.* 1938). I am unable to agree with many of the identifications, taxonomic placing, and anatomical findings in these

articles but detailed criticism will be withheld until a study of Pearse's specimens now under way has been completed.

LITERATURE CITED

- BOCK, S., 1913. Studien über Polycladen. *Zool. Bidrag*, 2: 31.
- BOCK, S., 1924. Eine neue Stylochoplana aus Japan. *Arkiv. Zool.*, 16: 1-24.
- BOCK, S., 1925. Planarians. Parts I-III. Papers from Dr. Th. Mortensen's Pacific Expedition 1914-1916. No. 25. *Vidensk. Medd. fra Dansk naturh. Foren.*, Vol. 79: 1.
- DIESING, K. M., 1861. Revision der Turbellarien. Abtheilung: Dendrocoelen. *Sitzber. math. naturw. Klasse Akad. Wissensch. Wien*, 44 (Abtheilung I): 485.
- FREEMAN, D., 1933. The polyclads of the San Juan region of Puget Sound. *Trans. Am. Micros. Soc.*, 52: 107.
- GIRARD, CH., 1850. Several new species of marine planariae of the coast of Massachusetts. *Proc. Boston Soc. Nat. Hist.*, 3: 251.
- GIRARD, CH., 1853. Descriptions of new nemerteans and planarians from the coast of the Carolinas. *Proc. Acad. Nat. Sci. Phila.*, 6: 365.
- GIRARD, CH., 1893. Recherches sur les Planariés et les Némertiens de l'Amérique du Nord. *Ann. Sci. Natur.*, Ser. 7, 15: 145.
- GRAFF, L. VON, 1892. Pelagische Polycladen. *Zeitschr. wiss. Zool.*, 55: 189.
- HYMAN, L. H., 1939. Polyclad and acoel Turbellaria from Bermuda and the Sargassum. In press, Bingham Oceanogr. Foundation.
- KATO, K., 1937. Polyclads collected in Idu, Japan. *Japan. Jour. Zoöl.*, 7: 211.
- LAIDLAW, F. F., 1902. Marine Turbellaria. Fauna and Geography of the Maldive and Laccadive Archipelagoes. I, Part 3.
- LAIDLAW, F. F., 1903. On a collection of Turbellaria Polycladida from the Straits of Malacca (Skeat Expedition, 1899-1900). *Proc. Zoöl. Soc. London*, 1903, Part 1, p. 301.
- LANG, A., 1884. Die Polycladen. Fauna und Flora des Golfes von Neapel, Vol. 11.
- MERTENS, H., 1833. Untersuchungen über den inneren Bau verschiedener in der See lebender Planarien. *Mém. Acad. Impér. Sci. St. Petersbourg*, Ser. V, *Sci. math. phys. nat.*, Vol. 2.
- MÜLLER, O. F., 1776. Zoologicae Danicae Prodomus seu animalium Daniae et Norvegiae indigenorum characteres, nomina et synonyma imprimis popularium. Havniae.
- PALOMBI, A., 1928. Report on the Turbellaria. Zoölogical Results of the Cambridge Expedition to the Suez Canal. 1924. *Trans. Zoöl. Soc. London*, 22 (Part 5): 580.
- STIMPSON, W., 1857. Prodomus descriptionis animalium evertibratorum, quae in Expeditioni ad Oceanum Pacificum Septentrionalem, a Republica Federata missa Cadwaladero Ringgold et Johanne Rodgers ducibus, observavit et descripsit. *Proc. Acad. Nat. Sci. Phila.*, 9: 149.
- VERRILL, A. E., 1873. Report upon the invertebrate animals of Vineyard Sound and the adjacent waters, etc. *U. S. Comm. Fish and Fisheries, Comm. Rept. for 1871-72*.
- VERRILL, A. E., 1882. Notice of the remarkable marine fauna occupying the outer banks off the southern coast of New England, No. 7, and of some additions to the fauna of Vineyard Sound. *Am. Jour. Sci.*, 24: 360.
- VERRILL, A. E., 1892. Marine planarians of New England. *Trans. Conn. Acad. Arts and Sci.*, 8: 459.
- WHEELER, W. M., 1894. Planocera inquilina, a polyclad inhabiting the branchial chamber of *Sycotypus canaliculatus*, Gill. *Jour. Morph.*, 9: 195.

Early references not listed here will be found in Lang (1884).

EFFECTS OF COLCHICINE ON THE CLEAVAGE OF THE FROG'S EGG (*RANA PIFIENS*)

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The most striking biological effect of the alkaloid drug, colchicine, is its capacity, when applied in the proper concentration, to arrest mitosis in the metaphase (Dustin, 1934, 1935). Under certain conditions, especially in plants, this drug has also been shown to be effective in producing polyploidy, doubtlessly due to its ability to inhibit to a greater or lesser degree the orderly sequences of the mitotic process (Blakeslee and Avery, 1937; Nebel and Ruttle, 1938).

There have been a great many studies of the effect of physical and chemical changes in the external environment on the developing egg of the frog. Some of the early results obtained, especially by centrifuging (O. Hertwig, 1897), and by exposure to high temperatures (O. Hertwig, 1898), rather closely parallel the effects produced by treatment with colchicine. In the present report the observations are limited to the changes induced by relatively short exposures to the drug applied almost immediately after fertilization.

Methods

The eggs of *Rana pipiens* were obtained and inseminated by the method of Rugh (1934). After a quarter of an hour the eggs were flooded with water and in another quarter of an hour were separated by dissection but the jelly was not removed. They were then put into the colchicine solutions. In later experiments the solutions were made up and used in the dark, except for a red light, since colchicine is affected in solution by light (Lits, 1936). The experiments were conducted at 8° and 20° C.

Description

In the first series of experiments, the colchicine solutions were made up in concentrations ranging from 1:1000 to 1:1,000,000 and the eggs were left in the solutions continuously. In the 1:1000 solution all cleavage activity was suppressed, and eventually disintegration set in. In the 1:10,000 solution the first cleavage took place as in the controls, the second cleavage set in at the normal time but showed some irregularities.

Soon after this, the membranes of most of the eggs seemed to be tightly stretched, appearing as though an abnormal amount of water had been absorbed. The cleavage grooves became more and more shallow. No further cleavage took place, and four and a half hours after fertilization no evidences of cleavage were visible except the presence of light streaks on the animal hemisphere. These streaks, which were due to the absence of superficial pigment, coincided with the position of the cleavage furrows. They appeared to have been caused by the movement of pigment deeper into the egg in association with the development of the clefts.

In the 1:100,000 solution of colchicine, the first, second, and third cleavages frequently took place as in the controls. Shortly after the initiation of the third cleavage, many of the eggs showed diminished grooves, the others continued dividing as in the controls. On the following day, some of those in which the grooves were fading showed no trace of cleavage except the light streaks, while others had the animal pole region largely cellular with the vegetative hemisphere uncleaved. The proportion of cellular to non-cellular material varied considerably. These were all dead on the third day.

In the 1:1,000,000 solution, the early cleavage was like that in the controls, no fading being evident except possibly on the vegetative halves. The second day embryos varied from completely normal to those with a small cell cap in the region of the animal pole, the rest of the egg being uncleaved. These embryos also, even those that appeared normal, were dead on the third day.

It is evident from these preliminary experiments that the eggs varied considerably in their susceptibility to colchicine, and that with a continuous exposure it was relatively toxic. A 1:100,000 exposure for one hour was found to give a fair number of abnormal forms without too high mortality and even a concentration of 1:10,000 for the same length of time was also satisfactory, although the mortality was somewhat greater. Most of the eggs in both these concentrations gave rise to normal embryos. The following studies, then, were made on selected individuals showing the characteristic abnormalities.

It is extremely difficult to interpret fully many of the abnormalities obtained by the treatment with colchicine. It was not possible to secure an orderly and progressive series of developmental stages as in normal development. The wide range of susceptibility of the eggs to the drug and the diverse effects obtained make it necessary to consider each egg individually and attempt to relate the conditions in earlier cleavages to the morphological patterns found in the more advanced embryos. In general the segmentation of treated eggs becomes meroblastic, with the

cap of cells produced in the region of the animal pole resting upon a more or less completely undivided yolk.

Early Cleavage

Reference has already been made to the fading of cleavage. Observations on living eggs and sections show that it takes place first at the vegetative pole, and sometimes a furrow extending halfway down the egg is left (Figs. 1, 2, and 3), while in other cases no trace of the cleavage remains, or only a shallow depression may persist at the surface of the animal pole. In some in which no external groove is left a cleft inside the egg can be seen in sections. In rare instances the first cleavage may persist even on the vegetative hemisphere, though subsequent cleavages are restricted in their persistence to the animal hemisphere. The nuclei may go on dividing although there is no cytoplasmic division. In several cases in which the early cleavage furrows had completely disappeared, a curious sort of delayed or secondary cleavage appeared at about the time when the controls were in the blastula stage (three days at 8° C.). In such cases a few relatively small cells were outlined on the surface, and contained one or more large vacuolated nuclei. More similar nuclei appeared in the main body of the egg.

The significant result of the arrest or retardation of the early mitosis and the secondary fading of the early cleavage furrows, especially in the vegetative hemisphere, is the production of an early blastula in which definitive cells tend to be restricted to the animal hemisphere. The degree of restriction of cell cleavage is variable. In extreme cases only a small cap of cells is formed but all gradations from this condition to almost complete cleavage of the yolk of the vegetative half may be encountered.

Blastulae

In many embryos with only a small cellular area in the region of the animal pole a stage comparable to the blastula is not attained. The cells divide progressively but do not assume any regular pattern. They are usually loosely grouped above the undivided yolk mass without the formation of a definite segmentation cavity (Figs. 4 and 6). This condition was observed most frequently in embryos which had developed at the lower temperature, 8° C.

In others, a modified blastula, consisting of a cellular roof which is separated more centrally from the uncleaved yolk by a segmentation cavity or blastocoele, is formed. This cavity apparently may be secondarily enlarged by the progressive vacuolation of the adjoining yolk mass (Fig. 7). Below the cavity are the free nuclei of the yolk, becom-



ing larger, more abnormal, and more irregularly spaced toward the vegetative pole. They are usually surrounded by dense accumulations of pigment.

In embryos in which approximately half of the material is cellular the blastula is frequently more complete with a cellular floor of variable thickness. The roof is often differentiated into two layers comparable to the outer, epidermal and inner, nervous layer. Occasionally, however, part of the animal half may fail to undergo segmentation while a considerable amount of the yolk is cleaved. In such cases the degree of primary segmentation that may have occurred in the yolk is difficult to determine. A study of living animal-hemisphere embryos showed that the first two cleavages could often be seen on the surface of the vegetative pole and subsequently faded. Whether or not these cleavages

EXPLANATION OF PLATE

Figures 1 to 9 are photomicrographs taken at several magnifications. The magnification was varied in order to bring out significant details or to include sufficient area to show relationships. In all instances the solutions of colchicine were applied for one hour, beginning thirty minutes after insemination.

PLATE I

EXPLANATION OF FIGURES

FIGS. 1 and 2. Modification of early cleavage; 4 hours, 20 minutes after insemination; temperature 20° C.; colchicine, 1:10,000.

FIG. 3. Early cleavage; 5 hours, 20 minutes after insemination; temperature 20° C.; colchicine, 1:10,000. The plane of section is oblique to the polar axis giving a false impression of the degree of cleavage of the vegetative hemisphere.

FIG. 4. External view of late cleavage showing restriction of cleavage to the animal half of the egg; 72 hours after insemination; temperature 8° C.; colchicine, 1:100,000.

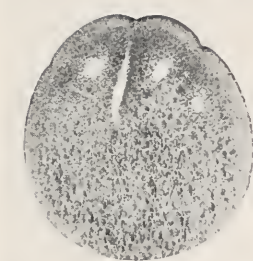
FIG. 5. Partial embryo; 48 hours after insemination; temperature 20° C.; colchicine, 1:100,000. From sections it appears that the first cleavage persisted and then cell division was restricted in one blastomere more than in the other.

FIG. 6. Late atypical cleavage; 72 hours after insemination; temperature 8° C.; colchicine, 1:100,000. Cleavage is sharply restricted to a cap of cells in the region of the animal pole. Large, numerous yolk-nuclei with concentrations of pigment about them are scattered through the uncleaved yolk.

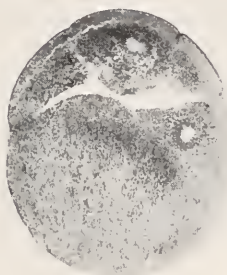
FIG. 7. Animal-hemisphere blastula; 48 hours after insemination; temperature 20° C.; colchicine, 1:100,000. Only the roof of the blastocoele is cellular. The floor is composed of unsegmented yolk which is highly vacuolated. The cellular configuration (on the right) possibly represents the beginning of gastrulation by involution.

FIG. 8. A transverse section of the upper half of an abnormal gastrula; 48 hours after insemination; temperature 20° C.; colchicine, 1:100,000. Neural plate, notochord, mesoderm and an incomplete layer of endoderm are present. The underlying yolk is undivided and ventrally an area of degeneration is seen.

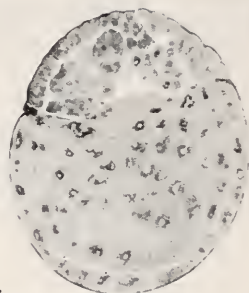
FIG. 9. An area from the cellular roof of a blastula; 48 hours after insemination; temperature 20° C.; colchicine 1:100,000. The nuclei of this embryo are of two distinct sizes. The smaller are of normal size. The larger may be due to tetraploidy.



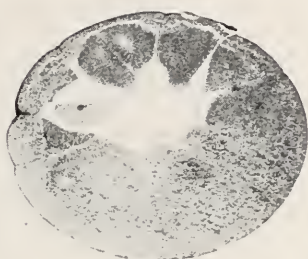
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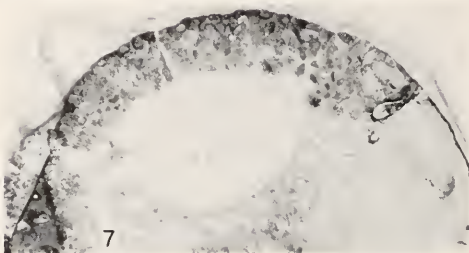
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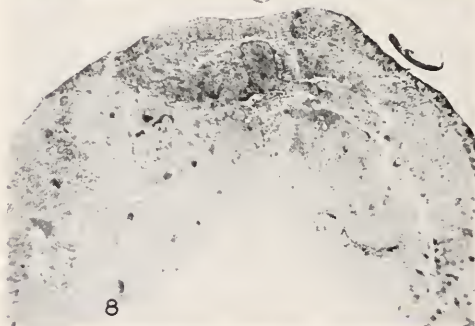
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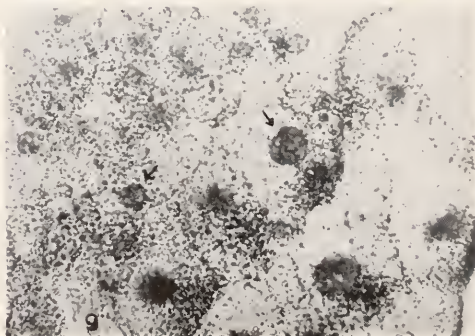
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9

PLATE I

actually passed completely through the yolk was not determined, but it seems fairly safe to conjecture that they had not. The formation of the cellular floor of the blastocoel may have occurred simultaneously with cleavage of the cells nearer the animal pole but there is evidence that in many cases the cleavage of the yolk occurs secondarily, probably through the progressive organization of cells from a region comparable to the germ-ring of normal embryos. As far as it is possible to judge from sections, the process of cellulation occurs around free nuclei directly adjacent to previously formed cells. In these regions the free nuclei are more likely to be normal in size and shape than those at a greater distance from the zone of definitive cells, i.e., nearer the vegetative pole. The cells formed are at first more or less spherical and sometimes extracellular yolk remains between them.

In many instances there was considerable disturbance of the distribution of pigment especially at the margin of the polar cap. Frequently the polar pigment was carried down as irregular streamers over the vegetative hemisphere leaving an irregular border between the animal and vegetative halves. In other instances the local disappearance of pigment on the surface of the animal hemisphere appeared to be due to its being carried deeper into the egg. Degenerative changes may be involved.

Gastrulae

It seems probable in the embryos in which segmentation is restricted to a cap of cells at the animal pole, that gastrulation could not occur. However, even in these, there is a tendency for the marginal cells of the cap to show a regional separation from the uncleaved yolk suggesting the initiation of gastrulation. In more complete blastulae, gastrulation may set in between the cleaved and uncleaved material. More often the yolk is cellular in the immediate region of the invagination. However, the evidence on the process of gastrulation is not conclusive. Externally the blastopore lip is straight and does not seem to be extended laterally very far around the circumference of the egg. In cases in which the yolk is not cleaved at the point of gastrulation the process seems to be limited to simple involution with the cells migrating to form a more or less continuous layer under the roof of the blastula. These cells are usually comparatively small and heavily pigmented, resembling those of the superficial epidermal layer. There does not seem to be any superficial down-growth in the region of gastrulation but laterally and anteriorly there is considerable overgrowth of a single layer of epidermal cells over the undivided yolk.

In embryos with cleaved yolk at the point of gastrulation there is usually a sac-like invagination which extends progressively into the blas-

tocoele and rests on the upper surface of the undivided yolk. In such cases the blastocoele is eventually obliterated and a small archenteron is established.

Later Development

Many embryos die after the initiation of the modified gastrulation, probably because of their inability to carry the process to any degree of completion. However, several survived in which gastrulation appeared to be represented primarily by involution. In these a disc-like blastoderm was developed which consisted of a neural plate with rudimentary neural folds, notochord, tissue occupying the position of dorsal mesoderm and a thin, sometimes incomplete layer of underlying entoderm (Fig. 8). A careful study of serial sections fails to yield any evidence of invagination. Caudally the epidermal cells, forming a free margin, rested directly upon the uncleaved mass of yolk. The yolk-filled tissue lateral to the notochord is interpreted as being derived by the progressive cellulation of the yolk adjacent to its junction with the margin of the blastula roof. The cells of the notochord and entoderm were relatively small and heavily pigmented. This, however, does not constitute an irrefutable argument for their origin by involution as the distribution of pigment is very irregular in these abnormal embryos. Furthermore, even in normal embryos the cells of this region contain considerable pigment, possibly related to their metabolic activity and more rapid multiplication.

Usually the segmentation of the animal hemisphere occurs uniformly and the partial embryos tend to be symmetrical. In some cases, however, the restriction of cleavage is unequal and asymmetrical embryos are produced. Such an embryo is illustrated in Fig. 5. It appears that in this case the first cleavage persisted and then the cell division of the region of the animal pole on one side was restricted more than on the other. Embryos do not survive beyond the neural fold stage.

Nuclear Size

In about half of the animal hemisphere embryos cells were found that were much larger than normal. Those generally occurred in groups and might be either epidermal or deeper-lying cells. They had correspondingly large nuclei (Fig. 9). Mitoses were observed but they were seldom favorable for chromosome counts due to the dense concentration of pigment granules about the spindle figure. Polar views adequately demonstrated that the number of chromosomes in the large cells was greater than normal but the exact ratio could not be established. It would seem quite probable that these represent tetraploid conditions. In

several embryos, one in particular, sectioned at late cleavage, the cells were of varied sizes, and the mitoses were very irregular. Eccentric, distorted monasters often at the sides or corners of the cells were frequent. Such conditions probably explain the variation in nuclear size.

In some cases, especially in eggs which had developed at 8° C., segmentation was restricted to a few superficial cells. In these, the adjacent area was characterized by regularly spaced, small centrospheres with associated asters and surrounded by pigment granules. Many of these were not associated with chromosomes or nuclear material and have been interpreted as cytasters.

Yolk and Free Yolk-nuclei

In the uncleaved portions of the egg the yolk underwent regular changes which were probably degenerative in character. The yolk platelets disappeared locally and large vacuoles were formed as the yolk products went into solution. Progressive coalescence of vacuoles produced large fluid-filled spaces which were distributed irregularly through the non-cellular portion of the egg (Fig. 7).

The free nuclei, especially in the areas more distant from the cleaved portion of the animal pole, became progressively larger and more irregular. In the earlier cleavage stages they appeared to undergo normal mitosis but in the blastula and later stages the mitoses became progressively abnormal and large, irregularly-lobed nuclei appeared. These nuclear complexes were usually surrounded by dense aggregations of pigment granules. Some nuclei are vacuolated and stain very lightly while others are massive and stain densely. These giant nuclei, however, are not characteristic only of colchicine-treated embryos. They were also observed by Hertwig (1897) in the meroblastic segmentation of the frog's egg under the influence of centrifugal force.

Summary

The treatment of the developing frog's egg with colchicine (concentrations from 1:10,000 to 1:100,000) in amounts sufficient to retard mitosis or cause a temporary arrest of the process, results in a varying proportion of the eggs exhibiting a meroblastic type of cleavage. The degree of restriction of cleavage to the animal-pole region varies from the formation of a small cap of cells to a condition in which cleavage may include almost the entire egg. The level of development and completeness of the embryo are directly correlated with the extent of restriction of cleavage. Such animal-hemisphere embryos may not be capable of attaining even a modified blastula stage. In many blastulae

only the roof of the segmentation cavity is cellular. The process of gastrulation is always greatly disturbed and in extreme cases may be limited to a modified involution. Frequently, neural plate with beginning neural folds, notochord, dorsal mesoderm and entoderm are differentiated. Embryos rarely develop beyond this level.

In many embryos there are scattered areas in which the cells are large and possess correspondingly large nuclei. These are tentatively interpreted as the result of tetraploidy, a phenomenon commonly produced in plants by colchicine. The free yolk-nuclei are characteristically abnormal.

LITERATURE CITED

- BLAKESLEE, A. F., AND A. G. AVERY, 1937. Methods of inducing doubling of chromosomes in plants by treatment with colchicine. *Jour. Hered.*, **28**: 393.
- DUSTIN, A. P., 1934. La radiothérapie et la chimiothérapie envisagées à la lumière des travaux sur les chocs caryoclasiques. *Jour. des Sci. Med. Lille*, No. 49, p. 561.
- DUSTIN, A. P., 1935. L'action de la colchicine sur les tumeurs malignes. Leeuwenhoek Vereeniging—IVieme Conférence, Amsterdam, p. 7.
- HERTWIG, O., 1897. Über einige am befruchteten Froschei durch Centrifugalkraft hervorgerufene Mechanomorphosen. *Sitzungsber. könig. Preuss. Akad. Wiss.*, Halbband 1, S. 14.
- HERTWIG, O., 1898. Ueber den Einfluss der Temperatur auf die Entwicklung von *Rana fusca* und *esculenta*. *Arch. mikr. Anat.*, **51**: 319.
- LITS, F. J., 1936. Recherches sur les réactions et lésions cellulaires provoquées par la colchicine. *Arch. Internat. Médecine Experim.*, **11**: 811.
- NEBEL, B. R., AND M. L. RUTTLE, 1938. The cytological and genetical significance of colchicine. *Jour. Hered.*, **29**: 3.
- RUGH, R., 1934. Induced ovulation and artificial fertilization in the frog. *Biol. Bull.*, **66**: 22.

EFFECTS OF 2, 4-DINITROPHENOL ON THE EARLY DEVELOPMENT OF THE TELEOST, *ORYZIAS LATIPES*

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The stimulating action of 2, 4-dinitrophenol on metabolism and oxygen consumption has been demonstrated for many organisms both embryonic and adult (Shoup and Kimler, 1934, luminous bacteria; Bodine and Boell, 1938, grasshopper embryos; Root and Etkin, 1937, toadfish; and others). It raises the metabolic rate of female rats (Halpern and Hendryson, 1935) but does not accelerate development in amphibian embryos (Cutting and Tainter, 1933; Dawson, 1938; Buchanan, 1938). In sea urchin eggs this and other nitro- and halophenols simultaneously increase respiration and block cell division (Krahl and Clowes, 1938). The physiological action is discussed by Bodine and Boell (1938).

In this investigation a study has been made of the effects produced by 2, 4-dinitrophenol on the development of the egg of the teleost, *Oryzias latipes*, (a) when continued exposure is begun at late cleavage to optic vesicle stages, (b) when the chorion is and is not pricked, and (c) when the egg is exposed to a lethal concentration for various periods of time.

This substance is highly toxic. In *Rana pipiens* (Dawson, 1938) it either retards, produces abnormalities or completely arrests development depending chiefly on the concentration. Among the abnormalities produced are: persistent blastulae, arrested early gastrulae, persistent yolk plugs and exogastrulae. These results are somewhat comparable to what others have obtained in amphibia with high temperature, thyroxine, x-ray exposure, and dilute Ringer's solution. Buchanan (1938) found that such respiratory effects as this substance may have do not compensate for the retarding effect of low temperature on the development of *Amblystoma*. The effects on gastrulation are especially interesting because of the numerous studies of exogastrulation which have been made in both amphibia and echinoderms.

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MATERIALS AND METHODS

Oryzias latipes (*Aplocheilus latipes*) is a small oviparous cyprinodont teleost, measuring about one inch in length, which is also known as the Japanese Medaka. This fish breeds well in the balanced aquarium and can stand low temperatures. Its small, colorless, transparent eggs are laid almost daily over long periods and remain attached to the cloacal region of the female until they are brushed off on the aquarium vegetation. The attachment occurs by the numerous processes peculiar to the egg chorion. Females lay from two or three to as many as twenty-five or more eggs at one time. If the eggs are regularly removed, they will continue to lay for several months. Otherwise the presence of the eggs appears to retard and later to inhibit egg production. Development is rapid and hatching occurs about fourteen to eighteen days after laying. Several factors may be responsible for this variation in hatching time, i.e. temperature possibly, inherent variation in developmental rate of embryos from the same and different females, and the time the eggs are laid in the reproductive period. At the end of the first day after laying, the eggs are in a late cleavage stage. The optic vesicle stage is reached during the second day and on the third to fourth days the heart is beating and differentiation is well advanced.

For the present study a 1 per cent stock solution of 2, 4-dinitrophenol was made according to the method of Halpern and Hendryson (1935), and sufficient amounts added to spring water to make the desired solutions. Concentrations of 1:10,000 up to 1:1,000,000 were used, but the most significant results appeared at concentrations from 1:40,000 up to 1:200,000. For the most part the embryos remained in the solutions for the entire experimental period. Eggs were also placed in a known lethal concentration for the late cleavage stage (1:10,000) and samples removed at intervals thereafter to fresh water. Since the egg is surrounded by a chorion, several studies were made in which the chorion was pricked. All experiments were made at room temperatures. Exposure was begun at four developmental stages; early and late cleavage, closure of the blastopore, and optic vesicle.

EXPERIMENTAL

In general the most conspicuous effect upon development is a retardation in both growth and differentiation which is proportional to the length of exposure and to the concentration of the drug, but there is variation in different individuals of a culture as some are more susceptible than others. No evidence of any stimulation was seen either in the developmental rate or in the hatching time nor were any cases

of exogastrulation obtained. The results obtained in one experiment are shown in Table I which is a typical example of the many that were

TABLE I
Effect of 2, 4-dinitrophenol on early development of *Oryzias latipes*
(Late cleavage stage)

Experiment	Concentration	Age in days	Number dead	Average heart-beat (No. of secs. for 50 beats)	Heart-beat (low and high)	Temperature when examined	Comments	Hatching time
21	Control	7	None	28.7	24 34	25° C.		1 in 18 days 1 in 21 days 5 in 25 days
	1:40,000	7	None in 8	35.2	33 42	25° C.	Markedly retarded. Few have no circulation and rudimentary heart.	All dead 13 days after laying.
	1:80,000	7	1 in 14	31.2	27.5 34.5	25° C.	3 retarded, others like control.	2 dead in 22 days, all dead in 25 days, none hatched.
	1:120,000	7	None in 15	29	26 32	25° C.	6 retarded	2 in 19 days 2 in 21 days 1 in 22 days 2 in 25 days All died soon after hatching.
	1:160,000	7	None in 14	28.6	26.5 31	25° C.	No retardation	1 in 18 days 8 in 19 days 2 in 21 days 1 in 25 days 2 died
	1:200,000	7	1 in 7	28.4	27 30	25° C.	No retardation	1 in 18 days 5 in 25 days

tried. Concentrations greater than 1:10,000 inhibit all further development beyond possibly a few cleavages, while in a solution of 1:160,000 development is normal and the embryos hatch about the same time as the control. Although development occurs in concentrations greater than 1:90,000, it is very retarded, causing early deaths and no hatching. The effect of the drug is cumulative with time and retardation becomes increasingly evident with continued exposure to strong concentrations. These observations confirm those of others on the high degree of toxicity of this substance.

Except for the difference in the effective concentrations and the formation of certain abnormalities, the effects of 2, 4-dinitrophenol on the development of this fish closely duplicate those found by Dawson on the frog, and by Solberg (1938) for *Fundulus* embryos exposed to X-rays. The survival of the embryos is directly correlated with their

sensitivity to the drug. It is generally difficult to duplicate the results of one experiment with eggs taken from the same or different females on successive days. Another comparable observation is that temporary developmental inhibition does not necessarily cause irreversible injury, providing the exposure is not too long.

Both the controls and the experimental animals were run at the same temperature. This was always that of the laboratory and it varied between 24° and 27° C., with an average around 25°. This slight variation probably did not influence the action of the drug since Dawson found that in the frog the results were apparently not greatly modified by temperature. However, a large proportion of his animals showed arrested and abnormal development at 6° to 12° C. This is far below the temperature range of the present study. In *Bufo*, Buchanan found that the toxicity is higher at 21° than at 6° C.

The heart and extra embryonic vascular system are particularly sensitive. Since the rate of heart beat corresponds roughly to the concentration and length of exposure, it serves as a check on the other effects observed. However, no stimulation was observed. Concentrations greater than 1:130,000 progressively slow up the rate and amplitude of the heart beat and also provoke structural irregularities. Deformities of the heart lead to interrupted and poor circulation which in turn provokes other abnormalities as development progresses. These embryos die early. In concentrations greater than 1:40,000 the extra-embryonic circulation never becomes established and the heart is rudimentary although other embryonic differentiations may take place, i.e. nervous system, sense organs, body form, and sometimes somites. Blood corpuscles develop in all embryos but the quantity is less in the more retarded types. Blood vessels are also affected as, in the extra-embryonic area especially, some may be distended with blood while others remain unconnected.

Deformities in the myotomes and a reduction of their number are of common occurrence. The number may especially be reduced in the tail. Here the irregularities bring about shortening and curious shapes and positions of the tail. It often becomes bent at odd angles, twisted, curved or shortened. The tail fins may fail to develop but typical paired appendages are usually present in less abnormal cases. Embryos of these types did not hatch.

The nervous system and sense organs exhibit a variable response to the drug. In the stronger solutions some development stops at a neural tube stage while other embryos show the presence of the optic vesicle with but slight indications of the brain. In others the nerve cord is shortened or deformed. Often the neural canal is filled with a mass

of cells. Different regions of the brain are often missing, enlarged or abnormal on one side or the other. Very little effect is observed on the ears. The optic vesicles often fail to invaginate or show a variable amount of pigmentation. A size difference is also noticed as well as a malformation of the lens.

No particular type of teratological embryo is produced by dinitrophenol, but the production of uniform abnormalities is fairly consistent. Gastrulation either occurs normally, is retarded, or is entirely inhibited. If the exposure is not too long a few of the retarded embryos

TABLE II

Sensitivity of different developmental stages to a lethal solution of
2, 4-dinitrophenol (1:10,000)

Stage	Length of exposure before transfer to fresh water					Development during exposure
	4 hrs.	10 hrs.	21 hrs.	29 hrs.	45 hrs.	
Early cleavage	Retarded in size and development.	Small optic vesicle or rudimentary nerve tube. Some dead.	Dead			None
Late cleavage	Retarded in size and development.	Small optic vesicle stage.	Small optic vesicle stage.	Dead		None
Closure of blastopore	Slight retardation in some.	Smaller and thinner embryos. Slight pigmentation of eyes.	No development beyond optic vesicle or young non-pigmented optic cup stage. Rudimentary heart. No circulation.	No development beyond young optic vesicle stage.	Dead	None. Perhaps slight increase in cell number.
Optic vesicle	No effect		Slight retardation. Slower heart rate.		Marked retardation. No heart action. 96 hrs. exposure did not inhibit development.	Yes, but at a slower rate.

The above results are those seen after the embryos had been in fresh water for 3-4 days following exposure.

are able to complete gastrulation and to continue development in fresh water. Otherwise they give cases of persistent yolk plug which also occur in some instances during the experiment. Subsequent development is very atypical and death soon ensues. The inhibited blastulae do not increase in size and development goes no farther except for a few irregular cleavages especially noticeable at the periphery. None of these are able to gastrulate in fresh water. Unlike the frog, no living cases of exogastrulation were seen.

The results of the study of the relative sensitivity of several developmental stages to dinitrophenol are summarized in Table II. They show that the sensitivity decreases at least from the early cleavage stage to the optic vesicle stage and in the latter case development is able to continue, but more slowly, in a lethal solution which quickly kills the earliest stage tested.

DISCUSSION

The resistance of fish embryos to the experimental conditions described in this study varies at different stages. At the optic vesicle stage they are much less sensitive to 2, 4-dinitrophenol than at the early cleavage stage. Solberg (1938) has likewise observed this to be true of *Fundulus* embryos exposed to X-rays. The earlier in development that the embryos are treated, the greater are the deformities. During the gastrula stages there is again a slight increase but thereafter the sensitivity declines rapidly and larger doses are required to modify development. Changes also occur in the sensitivity of different organs of the fish embryo to X-rays and dinitrophenol. As differentiation progresses the organs become more resistant; the time depends on the time of appearance and the rate of differentiation.

There was no special difference noted in the adjustment of early and late cleavage, or even older stages to the experimental solutions. Except for the question of sensitivity, early and late stages gave almost comparable effects. Dawson (1938) found this the case in his study of the effects of dinitrophenol on the development of *Rana pipiens* when treatment was begun at the 2-cell and early blastula stages. However, under the influence of high temperature, Hoadley (1938) found that the behavior of frog eggs is different immediately after the first cleavage from what it is at the 128-cell stage. There was a more complete adjustment to the new conditions on the part of the first group.

The suppression of cell division by dinitrophenol appears to be a common phenomenon. First reported (Krahl and Clowes, 1938) for sea urchin eggs, it has subsequently been found to occur in the frog (Dawson, 1938) and in the present study on the fish. At first this effect is hardly noticeable but with continued exposure mitosis is almost suppressed. The rate of this disturbance depends on the concentration of the drug. At one extreme development may be stopped at such an early stage as the optic vesicle, while at the other quite typical embryos may develop. However, the latter are noticeably smaller than the control, die sooner, and never hatch. Solberg (1938) finds that following X-ray exposure, the mitotic index of *Fundulus* embryos changes which accounts, at least in part, for the changes in sensitivity. The close

parallelism of the two changes during early development seems to him to be more than coincidental.

The chorionic membrane of the *Oryzias* egg is permeable to dinitrophenol but some difference is noted in the effective concentrations between cases of whole membranes and those in which the chorion is pricked. Even further differences might be noted if this membrane were entirely removed. The drug evidently penetrates slowly and higher concentrations and longer exposures are necessary for the intact membrane. This may account for the difference in the effective concentrations reported for the frog and *Bufo*.

In vertebrates, studies of exogastrulation have been limited thus far to the amphibia. The period of gastrulation is a critical one. Hoadley (1938) has shown that under the influence of supra-maximum temperatures early gastrulae and very late blastulae are unable to complete gastrulation and die. Early blastulae form giant blastulae which never undergo gastrulation. Many other environmental conditions are known to affect or modify amphibian gastrulation (cf. Dawson, 1938); dilute Ringers solution, X-ray exposure, thyroxin. Dawson obtained persistent blastulae, arrested early gastrulae, persistent yolk plugs and exogastrulae through the use of 2, 4-dinitrophenol. These results show that exogastrulation in amphibia is no more of a specific reaction to any particular agent than has been found to be the case among echinoderms (Child, 1936). They have all followed instances of developmental disturbance and arrest.

In the present study no cases of living exogastrulae were seen. Either gastrulation was inhibited entirely and the persistent blastulae remained as such until cytolysis and death occurred or gastrulation was retarded or stopped during the process. Cases of persistent yolk plug occurred occasionally. In all these interrupted types, the origin and the differentiation of the organ rudiments and even body formation were restricted. There was no indication of a differential effect on gastrulation alone. It would have been interesting to see what form exogastrulation would take in a telolecithal egg.

Certain types of abnormalities occurring in the embryos under the influence of 2, 4-dinitrophenol have been reproduced in fish by other agents and in the amphibia by various agents. For example, in *Fundulus heteroclitus* Solberg (1938) obtained comparable effects with X-rays on the development of the circulatory system, myotomes, nervous system, etc. Reference should be made to Dawson (1938) for work on the frog embryo. In general no special type of teratological embryo was produced but results were fairly consistent. As Dawson says, none of the effects obtained by treatment with dinitrophenol can be inter-

puted as being produced specifically by the drug but are such as might follow any treatment which disturbs the orderly processes of development and differentiation. If the increase in metabolic rate is one of the factors involved it is difficult to correlate this idea with the results secured by X-rays and by other agents tested on amphibia.

From the present study it would appear that 2, 4-dinitrophenol does not stimulate gastrulation or development in *Oryzias latipes* and, in the effective concentrations employed, it acts like any toxic agent. As such it retards, inhibits and disturbs normal orderly developmental processes in proportion to the concentration and length of exposure. Its effects are cumulative with time. Various concentrations which did not produce any observable defects caused no reduction in the hatching time. This confirms in the fish the observations of others that the drug does not accelerate development or metamorphosis in amphibian embryos (Cutting and Tainter, 1933; Dawson, 1938; Buchanan, 1938).

Dawson suggests that the increase in the metabolic rate may be one of the factors involved in the effects produced by 2, 4-dinitrophenol on the frog embryo since they resemble those caused by thyroxin (Bauermann) and high temperature. According to Bodine and Boell (1938) the toxic effect may be due to the increased accumulation of toxic metabolic products of which the organism is unable to rid itself. In this connection it is interesting to note the conclusion of Lindahl (1936) from his study of the effect of SO_4 -deficient sea water on the development of *Paracentrotus lividus* (the European sea urchin), since results were secured which in some cases are comparable to those of the frog and fish. SO_4 -deficient sea water produces persistent blastulae, exogastrulae, ectoblastulae with cilia, etc. He considers that hydrocarbon metabolism dominates at the animal pole while protein metabolism dominates at the vegetal pole. The toxic action of this deficiency is caused by the accumulation of toxic phenolic derivatives coming from protein metabolism.

SUMMARY

A study has been made of the effect of 2, 4-dinitrophenol on the development of four embryonic stages of the teleost, *Oryzias latipes*, i.e. early and late cleavage, closure of the blastopore and optic vesicle stages. Both the sensitivity of the whole organism and constituent parts to the drug decreases with age and differentiation. The general effect is cumulative and proportional to the concentration and length of exposure. In addition to inhibitory and retardative effects, other developmental abnormalities include those of the heart and blood vessels, myotomes, nervous system, body shape, etc.

Concentrations of 1:10,000 up to 1:1,900,000 were used, but the most significant results appeared at concentrations from 1:40,000 to 1:200,000. When the chorion is pricked there is some difference in the effective concentrations. No evidence of any stimulation was seen either in the developmental rate or in the hatching time. Gastrulation was either inhibited or retarded and no examples of exogastrulation were seen. If exposure has not been too long, recovery takes place to a more or less extent in fresh water.

Some of the results of the present study have been duplicated in the amphibia by various agents and in fish by X-rays. In general no special type of teratological embryo was produced but the results are fairly consistent and reproducible. It does not seem possible to attribute the effects to a specific action of this drug and hence to increased metabolic and respiratory rates alone, unless the change produces toxic products of which the embryo is unable to rid itself.

LITERATURE CITED

- BODINE, J. H., AND E. J. BOELL, 1938. The influence of some dinitrophenols on respiratory metabolism during certain phases of embryonic development. *Jour. Cell. Comp. Physiol.*, **11**: 41-64.
- BUCHANAN, J. W., 1938. Developmental acceleration following inhibition. *Jour. Exper. Zool.*, **79**: 109-128.
- CHILD, C. M., 1936. A contribution to the physiology of exogastrulation in Echinoderms. *Arch. f. Entw.-mech.*, **135**: 457-493.
- CUTTING, C. C., AND M. L. TAINTER, 1933. Comparative effects of dinitrophenol and thyroxin on tadpole metamorphosis. *Proc. Soc. Exper. Biol. and Med.*, **31**: 97.
- DAWSON, A. B., 1938. Effects of 2, 4-dinitrophenol on the early development of the frog, *Rana pipiens*. *Jour. Exper. Zool.*, **78**: 101-110.
- HALPERN, S. R., AND I. E. HENDRYSON, 1935. Comparative effects of dinitrophenol and thyroid on pituitary-gonadal complex of female rats. *Proc. Soc. Exper. Biol. and Med.*, **33**: 263-265.
- HOADLEY, L., 1938. The effect of supramaximum temperatures on the development of *Rana pipiens*. *Growth*, **2**: 25-48.
- KRAHL, M. E., AND G. H. A. CLOWES, 1938. Physiological effects of nitro- and halo-substituted phenols in relation to extracellular and intracellular hydrogen ion concentration. *Jour. Cell. and Comp. Physiol.*, **11**: 1-20.
- LINDAHL, P. E., 1936. Zur Kenntnis der physiologischen Grundlagen der Determination im Seeigelkeim. *Acta Zoologica*, **17**: 179-365.
- ROOT, R. W., AND W. ETKIN, 1937. Effect of thyroxine on oxygen consumption of the Toadfish. *Proc. Soc. Exper. Biol. and Med.*, **37**: 174-175.
- SHOUP, C. S., AND A. KIMLER, 1934. The sensitivity of the respiration of luminous bacteria for 2, 4-dinitrophenol. *Jour. Cell and Comp. Physiol.*, **5**: 269-276.
- SOLBERG, A. N., 1938. The susceptibility of the germ cells of *Oryzias latipes* to x-radiation and recovery after treatment. *Jour. Exper. Zool.*, **78**: 417-440.
- WATERMAN, A. J., 1937. Effect of salts of heavy metals on development of the sea urchin, *Arbacia punctulata*. *Biol. Bull.*, **73**: 401-420.

EFFECT OF TEMPERATURE UPON SHELL MOVEMENTS OF CLAMS, *VENUS MERCENARIA* (L.)¹

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INTRODUCTION

Knowledge of the effect of temperature on the physiological activities of mollusks is essential to the solution of many problems of shellfisheries. Galtsoff (1928), in his studies of the effect of temperature on oysters, demonstrated its bearing on the problems of oyster culture and sanitary control of the oyster industry. His studies and those of Nelson (1921) and Hopkins (1931) added greatly to our knowledge of this phenomenon in relation to oysters, but little or nothing is known about other Pelecypoda. This study deals with the effect of temperature upon the shell movements of the hard-shell clam (*Venus mercenaria* L.).

In Pelecypoda, respiration and feeding are influenced by three factors, namely, the rate of activity of cilia of the gill epithelium, changes in size of the ostia, and by the movements of the shell-valves. It has already been shown (Galtsoff, 1928) that low temperatures induce hibernation in oysters. During the hibernation period the animal is unable to feed because of the disturbances of the gill mechanism, and because of closure of shells. It is of interest, therefore, to determine the temperature at which an animal enters into the hibernating stage and whether or not there is a definite correlation between the shell movements of a mollusk and the temperature of the surrounding water.

Experiments on the effect of temperature upon shell movements of the clam (*V. mercenaria*) were begun on November 17, 1934, and continued until July 29, 1935. They were conducted in a large outdoor concrete tank, 10 by 20 feet, at the laboratory of the U. S. Bureau of Fisheries in Milford, Conn. The tank was so constructed that the water entered it only at the last third of each flood tide and flowed out only partially at the ebb. Thus, the water in the tank was partly renewed twice every twenty-four hours. At low water stages the tank retained approximately 4,000 gallons of water. Because of this arrangement rapid changes in the temperature and salinity of water in the tank were rather uncommon. A roof over the tank protected it from the hot sun

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parts, a foundation for keeping an experimental animal in a desired position (*A*), and a Foxboro recorder (*B*). An animal (*f*) imbedded in a block (*g*) of sand-cement mixture was placed on a large round concrete base (*r*) and connected to a recording instrument by a rod (*d*) and a chain (*a*) which was attached to a hook (*o*) of the recording apparatus. A flat piece of cement with a hole in the center was sealed to the free shell-valve of a clam, and on it rested a fine metal pin (*e*). By moving the nut (*i*) along the bent rod (*h*) the weight of a structure connecting the animal to the recording apparatus could be very accurately counterbalanced. This arrangement made it possible to eliminate any unnecessary pressure which would be exerted upon the clam. The rod (*j*) was connected to a writing pen (*k*) which recorded each movement of a clam shell upon the chart. By moving the hook (*o*) along the rod (*j*) the record of valve movement on a chart could be increased or decreased, as desired. In these experiments the distance between the experimental animal and the recorder was about 10 feet. The depth of the water over the clams varied from 4 to 7 feet depending upon the stage of tide.

While the time recorder has already been employed by other investigators in studying the shell movements of mollusks (Galtsoff, 1928), the method of holding the animal in a desired position and attaching it to the recorder, as described in this paper, is a new one.

The main advantage of using the apparatus described here, in preference to those employed by other investigators, is that it considerably simplifies the experimental work by rendering the handling and replacement of animals very easy. By pulling up the cord tied to the ring (*b*) of the foundation unit, the entire lower portion of the apparatus can be quickly raised from the bottom and the animal examined, or new animals substituted for the old ones. All manipulations in placing the animal in position and connecting it to the recording machine are performed out of water. The use of the chain (*a*) eliminates long rods, which were commonly used by other workers.

At each change of chart on the recording apparatus the experimental animal was tapped lightly to compel it to close its valves tightly. After this was achieved, a base line (*n*) was drawn on the chart by rotating it 360°. Having a distinct base line, every movement of a shell, no matter how slight, could be distinguished on the chart (*m*).

RESULTS

Table I and Fig. 2 show the percentage of time the clams remained open at different temperatures. While subjected to very low temperatures (—1.0° to +1.9° C.) all the clams remained completely closed.

Altogether 56 records were obtained at such temperatures. At temperatures ranging from 2.0° to 2.9° C., with the exception of one case when an animal was open for about 5 hours, all the clams were inactive. A very slight increase in clam activities was noticed at temperatures ranging from 3.0° to 4.9° C. At the latter temperature the majority of experimental animals remained completely closed, but a few opened on several occasions, bringing the average of open time to 9 per cent. A sudden increase in clam activities was noted at 5.0° to 5.9° C., when the percentage of time open increased to 35 per cent. Such a pronounced increase in shell activities indicates that for many clams the critical hibernating temperature lies somewhere between 5.0° and 6.0° C.

TABLE I

Number of 24-hour records of shell activities obtained at each temperature ranging from -1.0° to 28.0° C., per cent of total time, and average time shells remained open at each temperature during 24-hour period.

Temperature	No. of records	Per-centage of total time shells opened	Average time shells opened during 24 hours	Temperature	No. of records	Per-centage of total time shells opened	Average time shells opened during 24 hours
° C.			hours minutes	° C.			hours minutes
-1.0-0.1..	18	—	—	14.0-14.9	8	88	21 7
0.0-0.9..	8	—	—	15.0-15.9	16	81	19 26
1.0-1.9..	30	—	—	16.0-16.9	6	78	18 43
2.0-2.9..	26	1	0 14	17.0-17.9	12	84	20 10
3.0-3.9..	30	4	0 58	18.0-18.9	6	89	21 22
4.0-4.9..	32	9	2 10	19.0-19.9	4	76	18 14
5.0-5.9..	22	35	8 24	20.0-20.9	12	86	20 38
6.0-6.9..	6	29	6 58	21.0-21.9	6	90	21 36
7.0-7.9..	12	65	15 36	22.0-22.9	10	86	20 38
8.0-8.9..	22	67	16 5	23.0-23.9	4	78	18 43
9.0-9.9..	22	74	17 46	24.0-24.9	16	69	16 34
10.0-10.9..	4	88	21 7	25.0-25.9	9	71	17 2
11.0-11.9..	8	85	20 24	26.0-26.9	6	83	19 55
12.0-12.9..	26	84	20 10	27.0-27.9	4	89	21 22
13.0-13.9..	14	71	17 2				
				Total	399		

At 7.0° to 7.9° C. all the clams were open some of the time, bringing the average time open to 65 per cent. From 8.0° to 10.9° C. a further increase in shell activities was shown. At 10.9° C. the animals remained open 88 per cent of the total time. As the water warmed from 11.0° to 27.9° C. no definite correlation between the temperature of the water and the length of time the shells remained open could be detected. Between these two points, the percentage of open time fluctuated between 69 and 90.

The individual differences displayed by the clams used in these experiments were quite significant. For example, while some of the animals exposed to temperatures of 5.0° to 5.9° C. remained open for the entire period of twenty-four hours, others did not open at all.

At low temperatures the clams may remain completely closed for several days. For example, one clam had its shell closed from noon on December 16, 1934, until noon on January 3, 1935, a period of 18 days. The temperature of the surrounding water during that period fluctuated from — 1.0° to 6.5° C. Another clam remained completely

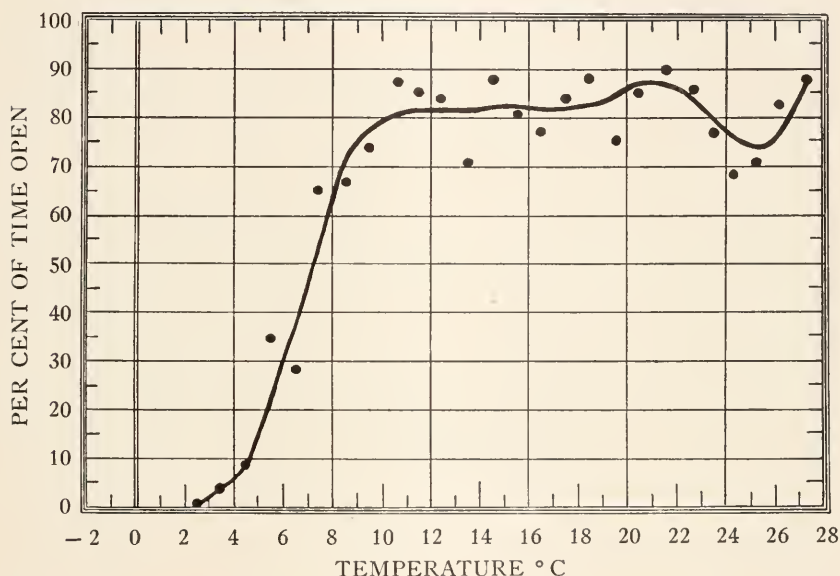


FIG. 2. Percentage of time clams remained open at different temperatures during 24-hour periods.

closed from March 8 to March 19, 1935. In several other instances the animals' shells remained closed for various periods extending from 3 to 6 days. In every one of these cases, the record of shell activity showed no deviation from the base line, thus indicating that not even a slight movement of the shells took place during such a prolonged period. Somewhat similar observations were made by Galtsoff (1928) on one specimen of *O. virginica* which remained tightly closed for 67 hours when left in cold water of a temperature varying from 0.5° to 1.6° C.

Galtsoff (1928), in his work on *O. virginica*, could not find a definite correlation between the effect of temperature and the time the shells of

oysters remained open. This was due, probably, to the fact that in his experiments the temperature of the water varied within the comparatively narrow range of 13.0° to 22.0° C. No systematic observations were performed at low temperatures. Similar conclusions could have been reached in this work if the observations had been confined to higher temperatures only. As has been mentioned already, there was no correlation found between the time the clam shells were open and the temperature increase from 11.0° to 27.9° C. If, however, the shell movements are examined at temperatures ranging from 0.0° to 11.0° C., the correlation between the rise in temperature and the gradual increase in duration of openness of the valves is noticed. This is especially evident within the range of 3.9° to 10.9° C., in which the average period of openness increases from 4 to 88 per cent (Fig. 2). Such an increase indicates that as soon as the water temperature gradually rises from the hibernation point to 11.0° C., the factors or conditions controlling the opening of the shells approach the optimum.

The present study, however, does not provide definite information as to the exact temperature at which the optimum conditions for opening of clam shells are reached. Within a 11.0° to 27.9° C. temperature range the shells are open from 69 to 90 per cent of the total time, but the time the shells of the animals remain open does not increase proportionally to the increase in temperature. The highest percentage of time open is recorded at temperatures 21.0° to 22.0° C. when the clams remained open 90 per cent of total time, or 21 hours and 36 minutes per 24-hour period. The average period of time the shells remained open at temperatures from 11.0° to 27.9° C. is 19 hours and 35 minutes. This figure closely resembles those obtained by some other investigators in their studies of oysters. Nelson (1921), basing his conclusions on the records of 3 oysters (*O. virginica*) kept under observation for 21 days, states that the animals remained open an average of 20 hours per day. Galtsoff (1928), from more numerous observations, concluded that the average period of time the shells of oysters remained open is 17 hours and 7 minutes per day. Hopkins (1931), working on *O. lurida*, found that the oysters were open and presumably feeding over 20 hours per day. From the study of the movements of clam shells and from observations of other investigators on oyster shell movements, it appears that under favorable conditions these pelecypods keep their shells open as long as possible.

Experimenting with *O. lurida*, Hopkins (1931) came to the conclusion that it is not so much the existing temperature of the water which determines how long the shells remain open as it is the changes in temperature which occur. This sensitivity of oysters to temperature

changes varies in an inverse manner with the temperature of the water. Thus, a small drop in temperature causes closure of the shell if the temperature is well below the optimum, but produces no effect if near the optimum. In clams no such effect of sudden changes in temperature could be detected. For instance, on December 2, 1934, between 2:00 and 3:00 P.M., the water temperature in the experimental tank rose

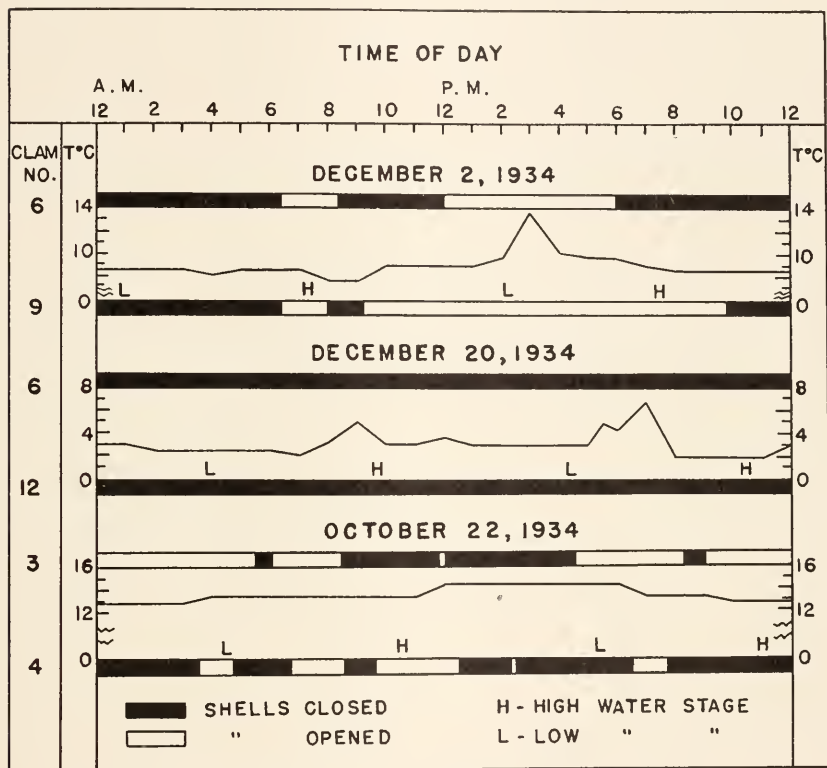


FIG. 3. Records of shell activities of clams and temperature of surrounding water during 24-hour periods. A sudden change in temperature (December 2 and 20, 1934) failed to cause the specimens to exhibit rapid closing or opening of shells. Shells closed and opened when temperature remained unchanged (October 22, 1934). No correlation between the tidal stages and shell movements could be observed. H—high-water stage, L—low-water stage.

from 9.5° to 13.5° C., and two hours later decreased to 9.5° C. (Fig. 3). Both of the experimental animals, whose shells were open, remained apparently undisturbed. In another case, on December 20, 1934, the water temperature in the experimental tank increased from 3.0° to 6.5° C. within two hours and then quickly dropped to 2.0° C. Neither of the two experimental animals showed any noticeable activities.

It appears that in *Venus mercenaria* the mechanism controlling the opening and closing of the shells is less sensitive to minor temperature changes than that of the oyster. In clams, the closing or opening of the shells is very often performed when the temperature of the surrounding water remains at the same point for some time. On the other hand, the closing or opening often occurred at decreasing temperatures, while again, in numerous other instances, these phenomena took place at increasing temperatures (Fig. 3, October 22, 1934). As far as can be judged by examining hundreds of the records obtained in this work, small changes in the temperature of the surrounding water do not directly influence the shell movements of clams.

During periods of openness the adductor muscles of the clam do not remain in the same position but relax and contract, indicating the changes in tonus level. Contraction of the muscles, although quite pronounced, seldom results in the complete closing of the shells. The data obtained help to answer the question whether there is a definite periodicity in contraction and relaxation of clam muscles, and, if so, whether such periodicity is affected by exposing the animal to different temperatures. Figure 4 represents the records of the shell movements of two experimental animals, Nos. 14 and 16, exposed to different temperatures ranging from about 0.0° to about 20.0° C. and having intervals of about 4.0° . The temperature of the water during these experiments fluctuated not more than $\pm 1.0^{\circ}$ C. Each record represents the activities of the clam for a period of 12 hours, from 6:00 P.M. until 6:00 A.M. The night period is chosen in preference to daytime because the changing of charts in the recording apparatus, usually made in daytime, slightly disturbed the animals. By taking the night half of a daily record, more reliable information is available. The muscles controlling the shells of clams relaxed and contracted, sometimes at very brief, and at other times at prolonged intervals (Fig. 4). It is difficult, however, to find a definite periodicity throughout any of the 12-hour periods shown in Fig. 4. Although during several brief intervals the movements of the shells were of a definitely periodic type and exhibited a rhythmic character, more often they occurred as unsystematic and inconsistent. In this respect the shell activities resemble those of oysters, in which, according to Hopkins (1936), partial closures of the shells are sometimes periodic, sometimes only occasional and unorganized.

Clams whose shell activities are graphically shown in Fig. 4 present an opportunity of comparing the behavior of two individuals subjected to identical environmental conditions. With the exception of the records obtained at the temperature of 16.0° C., all other records of the shell activities of these two clams were obtained simultaneously. At the tem-

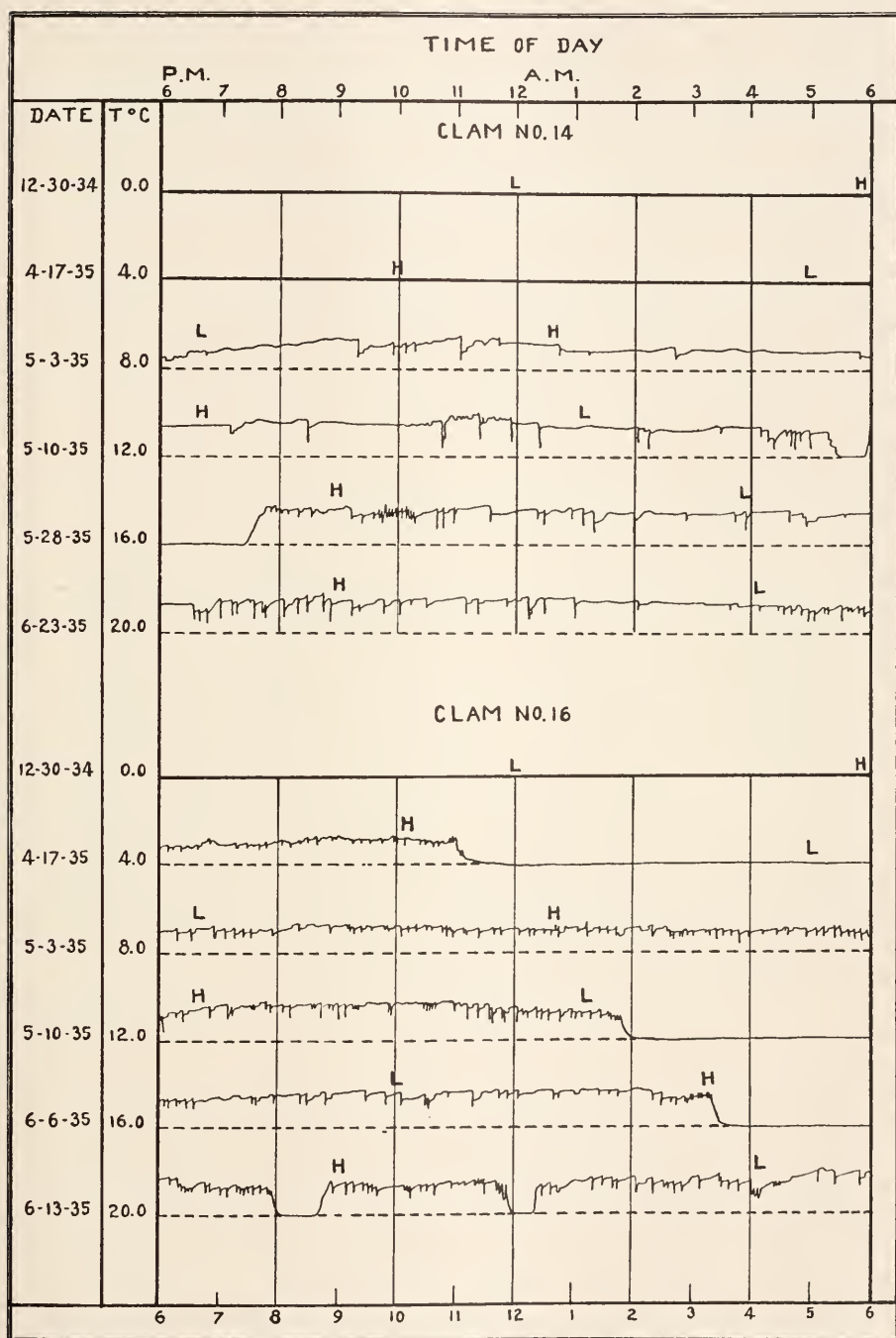


FIG. 4. Records of shell movements of two clams Nos. 14 and 16, exposed for 12-hour periods to different temperatures ranging from 0.0° to 20.0° C. Base line is indicated by series of dots. *H*—high-water stage, *L*—low-water stage.

perature of 0.0° C. both animals remained closed, thus exhibiting uniform behavior. At 4.0° C. one clam remained closed whereas the other was open part of the time. At higher temperatures both clams were active. The records show that there were considerable individual differences in the behavior of these animals although they were subjected to the same external environmental conditions. Such variability may probably be explained as due to internal causes.

In these experiments no definite correlation between the stage of the tide and shell movements of the experimental animals could be detected. This is demonstrated in Figs. 3 and 4 which show activities of clams at different tidal stages. The failure of the experimental animals to react

TABLE II

Percentage of time the shells of clams remained closed at daytime and night when exposed to different temperatures.

Temperature classes	Number of records	Number of clams	Average percentage of daylight closed	Average percentage of night closed
0.0- 4.9° C.....	7	3	67.0	74.0
5.0- 9.9° C.....	24	6	36.0	8.0
10.0-14.9° C.....	27	5	20.0	9.0
15.0-19.9° C.....	40	6	18.0	23.0
20.0-24.9° C.....	46	9	23.0	18.0
25.0-28.0° C.....	19	5	23.0	20.0

in a definite way to tidal changes is attributed to the fact that the tanks, where the animals were kept, are so constructed that they are filled at flood tide only and retain part of this water during the ebb stage. Therefore, fluctuations in temperature and salinity of water in the tank were not as great as those in the adjoining harbor.

The material on hand permits the answer to the question whether there is a correlation between opening and closing of clam shells and light and darkness. Nelson (1921) suggested that such correlation does exist for oysters. His conclusions were based upon oysters lying on a natural oyster reef subjected to the usual changes in temperature, salinity, pH and other factors found in open coastal waters. Galtsoff (1928), working in the laboratory under the fairly stable conditions of a relatively constant water supply and lesser differences between diurnal and nocturnal illumination, found no correlation between periods of closure and daylight hours. Webb (1930), working on *O. edulis*, found that the onset and termination of daylight have but little influence on the behavior of oysters as judged by their valve movements. Hopkins

(1931) observed the diurnal variation in the amount of time oysters (*O. lurida*) remained open, but this, according to his opinion, could be directly correlated with temperature fluctuation. In the present study 163 complete (24-hour period) records were examined. All other records showing that the animal was either open or closed for the entire period of 24 hours were not taken into consideration. The period of daylight was taken as the time between sunrise and sunset. After averaging all the data it was found that the clams kept their shells closed during 25 per cent of the total daylight, and 19 per cent of the total time of darkness. Thus, it appears that whereas there was no diurnal variation in shell activities of the clams, the animals were closed for a somewhat longer period in daylight than in darkness.

Records, mentioned above, were obtained between April 10 and July 29, 1935, thus covering the period of 110 days during which the environmental conditions of clam existence were gradually changing. In that span of time the temperature of the water increased from 3.7° to 27.5° C. Such a change in temperature, from below hibernation point for the majority of the clams to the maximum temperature of the year, offered an opportunity to determine whether the ratio between time of shell closure at daylight and at night varied with the rise in temperature as the season progressed. To answer this question all 163 records were arranged in six temperature classes, 5.0° apart. In referring to Table II, which shows the results obtained, it should be remembered that because the number of records is different for each temperature-class the direct comparison between classes is somewhat difficult. However, by studying this table it will be noted that the ratio of time the shells remained closed in daylight or at night varied considerably with the temperature. Such variation is especially significant at the temperature ranging from 5.0° to 14.9° C. At these temperatures the animals had their shells closed for a much longer time in daylight than at night. At present no explanation can be advanced for the occurrence of this phenomenon.

SUMMARY

1. A new apparatus, by means of which the shell activities of many bivalve mollusks can be measured and recorded, is described.
2. The analysis of 399 daily records of shell activities of 47 clams, subjected to temperatures ranging from -1.0° to 28.0° C., showed that the length of time which the animals remain open partly depends upon the temperature of the surrounding water.
3. For the majority of clams hibernation begins soon after the water temperature decreases to 5.0° and 6.0° C. At lower temperatures the

clams may remain completely closed for very long periods. No shell movements were exhibited, and no disposal of ejecta occurred.

4. Within the temperature range of 3.9° to 10.9° C., the average period of openness increased from 4 to 88 per cent of the total time, showing a correlation with the rise of temperature.

5. There was no correlation between the duration of openness of the clam shells and the temperature increase from 11.0° to 27.9° C. Within this temperature range the shells were open from 69 to 90 per cent of the total time, but the percentage did not increase simultaneously with the increase of water temperature.

6. The highest percentage of time open was recorded at temperatures 21.0° to 22.0° C., when the clams remained open 90 per cent of the total time, or 21 hours and 36 minutes per 24-hour period.

7. Small changes in the temperature of the surrounding water did not influence the shell movements of clams.

8. There appeared to be no definite periodicity in the clam shell movements. During brief intervals, the shell movements may be of a periodic type and exhibit a rhythmic character, but generally they appeared to be unsystematic and inconsistent.

9. There were considerable individual variations in the behavior of clams kept under identical environmental conditions.

10. Under the conditions of the experiments no definite correlation between the stages of tide and the shell movements of the animals could be detected.

11. The animals were closed for somewhat longer periods in daytime than in darkness.

LITERATURE CITED

- GALTSOFF, P. S., 1928. Experimental study of the function of the oyster gill and its bearing on the problems of oyster culture and sanitary control of the oyster industry. *Bull. U. S. Fish.*, 44: 1928 (1929): 1-39.
- HOPKINS, A. E., 1931. Temperature and the shell movements of oysters. *Bull. U. S. Fish.*, 47: 1-14.
- HOPKINS, A. E., 1936. Activity of the adductor muscle in oysters. *Physiol. Zoöl.*, 9 (4): 498-507.
- NELSON, T. C., 1921. Report, Dept. of Biol., New Jersey Agric. College Exp. Sta., for year ending June 30, 1920 (1921): 317-349. Trenton.
- WEBB, MARSHALL H., 1930. An apparatus for recording the valve movements and the extrusion of dejecta of oysters. *Jour. du Conseil*, 5 (3): 361-382.

THE ACTION OF CERTAIN DRUGS ON THE INSECT CENTRAL NERVOUS SYSTEM

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The purpose of this paper is to describe the effects of certain drugs on general reflex activity in insects, and to compare the pharmacology and physiology of the insect and vertebrate nervous systems. The literature reveals few positive observations of the effect of injected drugs on insects, though in general it seems that insects are less responsive than other invertebrates.

MATERIAL AND METHODS

The material for these experiments consisted of mature female praying mantids (*Mantis religiosa*) and mature cockroaches (*Periplaneta americana*). The various drugs used were made up in .6 per cent saline shortly before injection. The drugs were introduced by injection into the head capsule with a small syringe and fine hypodermic needle. Since the insect circulation is rather sluggish, there is only slight general distribution of an injected solution, and the site of injection determines to some extent the effect of a drug. An injection of .01 cc. into the head capsule appears to reach the supraesophageal and to some extent the subesophageal ganglion, but has little or no effect on the rest of the nerve cord. Records were kept in the form of written observations and 16 mm. moving pictures. Little attempt will be made to present quantitative results, since it is difficult to inject an exactly measured dose into an unanaesthetised insect, and there is no satisfactory way of quantifying the resultant change in behavior without giving false impressions.

EXPERIMENTAL

Strychnine

The normal posture and degree of activity of the mantis are variable, and have been described at length (Roeder, 1937). Injection of .02 mg. strychnine causes a slight drop in tonus and decrease in antennal vibration. This effect is very transitory, lasting only a few minutes. Larger doses of from .05 to .1 mg. result in a pronounced departure from the normal within 3 to 5 minutes. First the antennal vibrations become

slower and the movements of the mouth-parts very sluggish. With large doses movements of the head appendages cease entirely. The tonus of the trunk muscles drops, and the insect takes on a crouching posture owing to the sagging of the long prothorax. The degree of locomotor activity varies, though at first there is an increase, the insect walking slowly and restlessly. The movements of the legs are coördinated, and identical with normal locomotor movements. Owing to insensitivity of the head appendages and the crouching posture, the mantis is unable to climb up or over any obstacle, and since it moves in a straight line, it eventually becomes wedged in a corner or under some object. Mechanical stimulation of the antennae or photic stimulation of the eyes fails to produce any response, and the whole head appears to be quite insensitive. These symptoms: a drop in tonus, increased locomotor activity, and paralysis of the head appendages also follow destruction of the cerebral ganglia, therefore the effect of strychnine on the mantis central nervous system is entirely depressant. Experiments with cockroaches yielded essentially similar results. The insects became very quiet, and there was little mouth-part or antennal movement. Both mantids and cockroaches recovered completely from the effects of strychnine within an hour.

The effect of strychnine on these insects is then in direct contrast to its well-known effect on the vertebrate spinal cord, where it supposedly lowers thresholds between neurons in the cord, and is capable of turning an inhibitory process into an excitatory one. Many invertebrates show a comparable reversal of inhibition (Knowlton and Moore, 1917; Moore, 1918; Crozier and Federighi, 1924; and Crozier, 1927, 1930), though strychnine is recorded as having little or no effect on insects (Crozier, 1922; Crozier and Pilz, 1924). Many other papers are summarised in a review by Poulsson (1920). The only vertebrate organ which is depressed by strychnine is the heart (Poulsson, 1920).

Pilocarpin

This drug belongs to a group which has a specific stimulating effect on parasympathetic effectors (Kuntz, 1934). In vertebrates it increases salivary secretion, produces vasodilation, has a vagus-like effect on the heart, and increases contractility in the stomach and intestine, while its action is blocked by atropin (Dixon and Ransom, 1924). No record of its effect on the vertebrate central nervous system could be discovered.

If pilocarpin is injected into the head capsule of a praying mantis, it produces a state of great excitation. The minimum dose producing observable effect is about .01 mg. while doses of from .02 to .1 mg. bring about a change in behavior within thirty seconds. The walking legs become flexed, the prothorax raised, and the head ventrally flexed.

apparently due to an increase in tonus of the ventral neck muscles. There is an increase in amplitude and rate of antennal movement, and the mandibles are opened and shut, producing a clicking sound. There are spasmodic movements of the raptorial legs, consisting of flexions and extensions, and occasionally the whole body is swayed rapidly from side to side. All reflex activity increases except locomotion. Only three out of thirty insects showed any tendency to walk, either spontaneously or on stimulation. Recovery occurs rapidly and the insect is normal within an hour.

High muscle tone and inhibition of locomotor activity are typical of an insect with the right and left cerebral ganglia separated. This is interpreted as being due to increased activity of the tonus and inhibitory locomotor centres when released from the inhibitory effect of the contralateral ganglion (Roeder, 1937). Since pilocarpin when applied to the cerebral ganglia of an intact insect has the same effect as median division, it must have an excitatory action upon these centres. This can be substantiated by removing the cerebral ganglia prior to injection. An insect so treated walks rapidly in a crouching position. Since the cerebral centres are now absent the pilocarpin exerts its excitatory effect upon the locomotor centre in the subesophageal ganglion with consequent activity.

Pilocarpin has a comparable effect on cockroaches. A solution of the drug was injected into the head in doses of approximately .01 cc. A solution of 1:500 produced immobility and apparent paralysis but solutions of 1:500 to 1:5,000 caused intense excitation. It is impossible to give an adequate description of the behavior because so many things occur simultaneously. The mouth-parts and antennae are in continual movement, the head is moved from side to side and ventrally flexed, and cleaning movements are made with the legs, which are often abnormally extended. Occasionally there are violent cramps and spasms involving the whole of the body musculature and very rapid side-to-side swaying movements similar to those observed in treated mantids. More dilute solutions of 1:5,000 to 1:25,000 either have no noticeable effect or produce only a restlessness and general increase in activity. The above symptoms appear within one minute of injection and last for about thirty minutes. Recovery is usually complete. The excitation produced by pilocarpin seems to indicate a similarity between the nervous systems of these two insects and the vertebrate parasympathetic. This similarity leads to an investigation of the response to acetylcholine and eserine.

Acetylcholine and Eserine

Recent work has shown that acetylcholine acts as mediator between parasympathetic nerves and autonomic effectors, and between pre- and

postganglionic fibres in the autonomic ganglia. Naturally produced acetylcholine is destroyed very rapidly by the enzyme esterase, but it can be protected from destruction by a drug, eserine. By its protective action eserine renders autonomic effectors and postganglionic fibres more sensitive to introduced acetylcholine, and, by preventing the immediate destruction of naturally produced acetylcholine, can prolong the postganglionic response to a preganglionic stimulus (Cannon and Rosenblueth, 1937).

For these experiments cockroaches only were used, as mantids were not available. Acetylcholine hydrochloride was dissolved in .6 per cent saline, and injected into the head, in .01 cc. amounts. Strengths from 1:500 to 1:25,000 were used but in no case was any change in behavior noted. Since the acetylcholine was unprotected by eserine its effect may have been only transitory, and therefore would have escaped attention. In order to prolong the effect, if any, cockroaches were first injected with eserine. This drug had an unexpected effect, which was comparable with, but more pronounced than, that of pilocarpin. Eserine alone in dilutions of 1:250 to 1:500 produced an almost instantaneous spasm and immobility from which the insect did not recover, while solutions of 1:500 to 1:2,500 caused enormous reflex activity, which consisted of rapid antennal quivering, continuous mouth-part activity, movements of the head, great increase in extensor leg tonus and general spasms. Usually the insects did not walk, but one or two were so active that no observation could be made. Solutions more dilute than 1:5,000 either had no effect or produced only a slight restlessness.

It can be seen that this strong eserine effect made it impossible to test the effect of acetylcholine protected by eserine. Though the presence of acetylcholine in the nervous system of these insects has not been demonstrated, the action of eserine could be explained by its presence.

Atropin

The specific action of this drug is to block transmission of impulses in autonomic ganglia and to parasympathetic effectors. It also renders these structures insensitive to parasympatheomimetic substances such as acetylcholine and pilocarpin (Cannon and Rosenblueth, 1937). From the foregoing experiments it might be expected that atropinised insects would fail to respond to pilocarpin, and would also show a decreased reflex activity. The answer to the second question is somewhat doubtful. Atropinised cockroaches remain very inactive for long periods, though they are able to respond if disturbed. Cockroaches were first brought into a condition of great activity with .01 cc. of 1:500 pilocarpin, and

then subjected to an injection of atropin. This failed to decrease the pilocarpin effect to a noticeable extent. If the insects were first injected with atropin and then given an injection of 1:500 pilocarpin, they showed either normal or decreased activity in eight out of ten cases. Two insects showed a very brief phase of excitation, lasting a few seconds, and then became inactive, but in no case was there the prolonged and intense reflex activity which occurs if such a dose of pilocarpin is administered alone. Therefore, previous treatment with atropin protects cockroaches against the pilocarpin effect, just as it does in the vertebrate parasympathetic.

CONCLUSIONS

The following conclusions seem justified:

(1) The effect of strychnine on the reflex activity of the mantis and cockroach indicates that the central nervous systems of these insects and vertebrates differ in certain respects.

(2) The action of pilocarpin, eserine, and atropin on the reflex activity of these insects indicates that there is a pharmacological similarity between their nervous systems and the vertebrate parasympathetic.

It must be recognized that the experiments outlined above are suggestive rather than conclusive, since the methods used are in many ways unsatisfactory. First, there is no way of knowing the exact concentration of drug actually reaching the cerebral ganglia, partly because it is not an easy matter to inject small exact amounts into a struggling insect, and partly because of the sluggish and casual nature of the insect circulation. Second, there is no way of telling exactly what structures are being reached by the injected drug. From information available on the functions and interrelations of the cerebral ganglia in the mantis (Roeder, 1937) it seems highly probable that these structures are being directly affected. The great activity of the mouth-parts indicates that the subesophageal ganglion is also affected to some extent, though it is hard to say to what extent the drugs are affecting sense organs and neuro-muscular junctions. Third, observation of changes in the general reflex activity is a complex and unsatisfactory criterion. However, a satisfactory technique avoiding some of these difficulties has been developed, and it is hoped that more extended conclusions can be submitted in a later paper.

The similarity of the insect central nervous system and the vertebrate parasympathetic immediately suggests the presence of a chemical substance such as acetylcholine, which would serve as mediator in the transfer of impulses from neuron to neuron in the central nervous system. No proof of the presence of such a substance is presented here,

though the action of eserine is suggestive. Acetylcholine unprotected by eserine produces no noticeable effect when injected, but an insect is capable of such rapid and brief responses that one would expect very rapid destruction or removal of any substance mediating the transfer of impulses.

The question as to whether these findings apply generally to insects cannot be given an adequate answer. It has been reported that strychnine produces little or no excitation in caterpillars (Crozier and Pilz, 1924), while pilocarpin raises sensory thresholds in the same insects (Crozier, 1922), while crustacea (Clarke and Wolf, 1932; Viehoveer and Cohen, 1937) and many invertebrates respond to strychnine in a vertebrate-like manner. It would seem that a generalization with respect to insects must await a further investigation.

SUMMARY

1. Adult praying mantids and cockroaches were injected in the head with solutions of various drugs, and the changes in general reflex activity were recorded.

2. Strychnine caused a decrease in reflex activity in both insects, large doses causing complete cessation of antennal and mouth-part movement. This is contrary to its effect on the vertebrate spinal cord.

3. Pilocarpin and eserine bring about an increase in mouth-part and antennal movement, head movement and extensor leg tonus, and produce spasmodic twitching and general contraction of the body musculature. In the case of pilocarpin this effect is prevented by atropin.

4. Acetylcholine alone has no detectable effect on activity. Its effect, if any, when injected with eserine is masked by the excitatory effect of eserine.

5. It is concluded that, in their reactions to drugs, the nervous systems of the two insects studied show little similarity to the vertebrate central nervous system, but considerable similarity to the vertebrate parasympathetic.

LITERATURE CITED

- CANNON, W. B., AND A. ROSENBLUTH, 1937. *Autonomic Neuro-effector Systems*. The Macmillan Company, New York.
- CLARKE, G. L., AND E. WOLF, 1932. The mechanisms of tropistic reactions in *Daphnia* and the strychnine effect in *Daphnia*. *Jour. Gen. Physiol.*, **16**: 99-105.
- CROZIER, W. J., 1922. "Reversal of inhibition" by atropine in caterpillars. *Biol. Bull.*, **43**: 239-245.
- CROZIER, W. J., 1927. Galvanotropism and "reversal of inhibition" by strychnine. *Jour. Gen. Physiol.*, **10**: 395-406.
- CROZIER, W. J., 1930. Reversal of galvanotropism in echinoderms. *Jour. Gen. Psychol.*, **3**: 268-276.

- CROZIER, W. J., AND H. FEDERIGHI, 1924. Suppression of phototropic circus movements of *Limax* by strychnine. *Jour. Gen. Physiol.*, **7**: 221-224.
- CROZIER, W. J., AND G. F. PILZ, 1924. Central nervous excitation by alkaloids in insects. *Am. Jour. Physiol.*, **69**: 41-43.
- DIXON, W. E., AND F. RANSOM, 1924. Pilocarpin, physostigmin, Arecolin. Gifte welche bestimmte Nervenendigungen erregen. Handbuch der Experimentellen Pharmacologie 2 (2) 746-785. Herausgegeben von A. Heffter. Julius Springer, Berlin.
- KNOWLTON, F. P., AND A. R. MOORE, 1917. Note on the reversal of reciprocal inhibition in the earthworm. *Am. Jour. Physiol.*, **44**: 490-491.
- KUNTZ, A., 1934. The Autonomic Nervous System. Lea and Febiger, Philadelphia.
- MOORE, A. R., 1918. Reversal of reaction by means of strychnine in planarians and starfish. *Jour. Gen. Physiol.*, **1**: 97-100.
- POULSSON, H., 1920. Die Strychningruppe. Handbuch der Experimentellen Pharmacologie 2 (1). Herausgegeben von A. Heffter. Julius Springer, Berlin.
- ROEDER, K. D., 1937. The control of tonus and locomotor activity in the praying mantis (*Mantis religiosa* L.). *Jour. Exper. Zööl.*, **76**: 353-374.
- VIEHOEVER, A., AND I. COHEN, 1937. Mechanism of strychnine. *Am. Jour. Pharm.*, **109**: 285-316.

SOME PROPERTIES OF SPERM EXTRACTS AND THEIR RELATIONSHIP TO THE FERTILIZATION REACTION IN *ARBACIA PUNCTULATA*¹

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Since the early work of Piéri (1899), numerous attempts have been made to isolate substances from sperm which could be shown to function in normal fertilization. Extracts and filtrates of sperm, prepared by a wide variety of methods, have consistently proved inactive as parthenogenetic agents. The literature of this subject has been reviewed by Sampson (1926), who points out that any positive results reported may be ruled out on the basis of technical or interpretive errors. Largely because of these discouraging results, the search for the hypothetical "fertilizing substance" of sperm has been abandoned during the past decade.

The purpose of this investigation was to study certain properties of sperm extracts obtained by heating and filtering sperm suspensions of *Arbacia*. The experiments deal with (1) the agglutinating action of sperm extracts on *Arbacia* eggs, (2) the relation of such extracts to egg-water ("fertilizin" of Lillie, 1913, etc.), (3) the effects of sperm extracts on the fertilizing power of sperm, the fertilizability of eggs, and the development of fertilized eggs, and (4) the action of sperm extracts as parthenogenetic agents.

MATERIALS AND METHODS

The experiments were carried out during the summers of 1934, 1937, and 1938 on the gametes of the sea-urchin, *Arbacia punctulata*. Eggs and sperm were obtained by the method described by Just (1928). After cutting out the lantern and peristome with scissors, the coelomic fluid was washed away with sea water and the urchin placed aboral side down over a Stender dish and allowed to shed. Mixed "dry" sperm from several males was kept in a test tube until required and mixed shed eggs were washed and placed in a small volume of sea water. Where large amounts of material were needed or when the

¹ A thesis submitted in partial fulfillment for the degree of M.D., Yale University School of Medicine, New Haven, Conn.

animals shed poorly, the ripe gonads were removed with forceps, washed, and strained through cheesecloth. All instruments and glassware were rinsed thoroughly in tap and sea water before use to prevent chance contamination of eggs with sperm.

In preparing jellyless eggs, shed ("normal") eggs were treated with a mixture of 1.4 cc. N/10 HCl in 50 cc. sea water, washed, and examined in Chinese ink to insure the absence of jelly (Lillie, 1915a). This procedure removes the jelly from 90-100 per cent of the eggs without harming them.

Sperm extracts were prepared as follows: Sperm suspensions, made up in 10-cc. portions by adding a measured volume of dry sperm to sea water, were heated one minute over a small Bunsen flame. The albuminous sperm coagulum was removed by filtration through No. 5 Whatman filter paper. The filtrate, or sperm extract, was considered to be equal in concentration to that of the sperm suspension from which it was obtained. Thus a 10 per cent sperm extract was prepared by heating and filtering 1 cc. sperm in 9 cc. sea water.

In preparing and determining the fertilizin concentration of egg-water, Lillie's method (1914) was followed with slight modifications. Fresh unfertilized *Arbacia* eggs, which had stood at least fifteen minutes in a small volume of sea water, were lightly centrifuged and the supernatant fluid (egg-water) removed with a pipette. The presence of fertilizin was detected by adding a drop of egg-water to a drop of fresh 1 per cent sperm suspension and observing reversible agglutination of the sperm under the microscope. By making successive half-dilutions of the egg-water with sea water, a dilution is reached at which the sperm agglutinate for three to six seconds but above which no agglutination occurs. This dilution, by definition, contains one fertilizin unit and the fertilizin concentration of the original egg-water is thereby determined. For example, if an egg-water agglutinates sperm at 1/1600 dilution but not at 1/3200, the undiluted egg-water contains 1600 fertilizin units.

THE EGG-AGGLUTINATION REACTION

When a few drops of *Arbacia* eggs are added to a small quantity of sperm extract in a tube, a striking reaction occurs. As each drop falls it sets into a dense red mass which remains suspended from the surface of the solution by a thin ribbon of coagulated eggs (Fig. 1, *A*). When the tube is shaken the ribbons coalesce to form a loose clot of eggs which usually floats at the surface of the extract (Fig. 1, *B*). A similar quantity of eggs, shaken in heated water as a control, forms

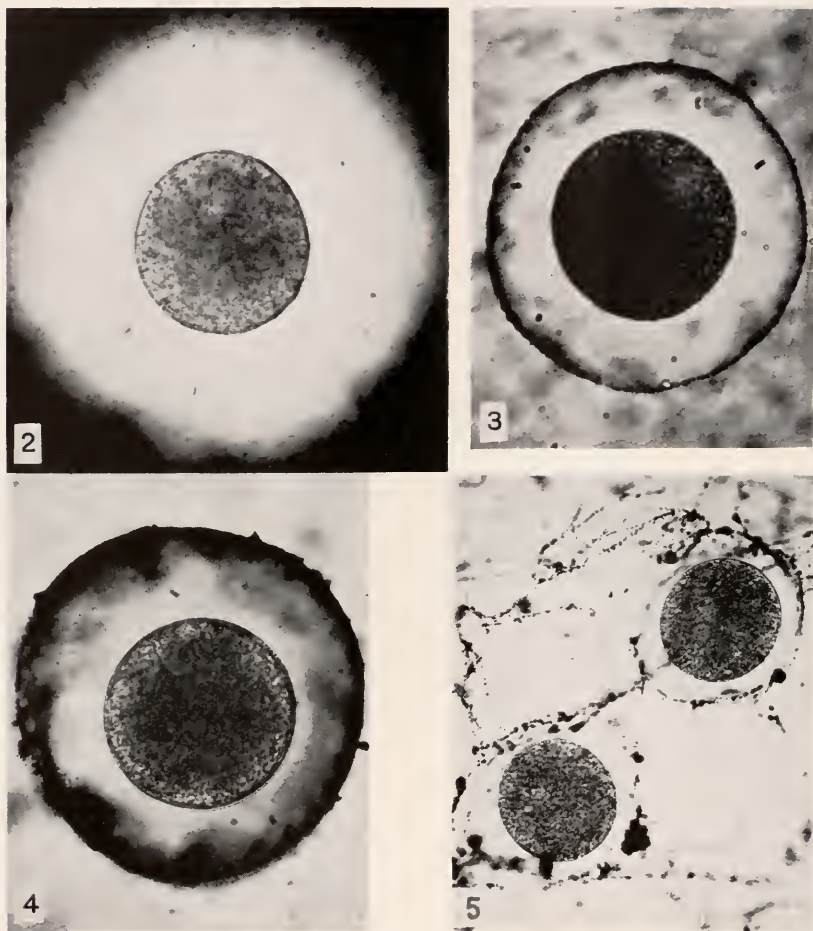


PLATE I

Microscopic appearance of the egg-agglutination reaction. All eggs photographed in Chinese ink.

FIG. 2. Normal *Arbacia* egg in heated sea water surrounded by a wide zone of clear, colorless jelly. $\times 430$.

FIG. 3. A similar egg after five minutes exposure to a 5 per cent sperm extract. Note that a distinct agglutination membrane has appeared at the periphery of the jelly. $\times 430$.

FIG. 4. A similar egg after thirty minutes exposure to a 5 per cent sperm extract. The agglutination membrane has become denser but the jelly immediately in contact with the cortex remains unaffected. $\times 430$.

FIG. 5. Strands of agglutinated jelly connecting eggs in 5 per cent sperm extract. $\times 215$.

a homogeneous suspension which slowly settles (Fig. 1, C). Subsequent agitation of each tube causes an increase in the density of the egg-masses in the extract, while the eggs in sea water redistribute themselves through the solution and again settle to the bottom of the tube. We will call this phenomenon the "egg-agglutination reaction," and the inciting substance present in sperm extracts, the egg-agglutinating substance. These terms are used in a purely descriptive sense without implying an analogy to immunological agglutinations.

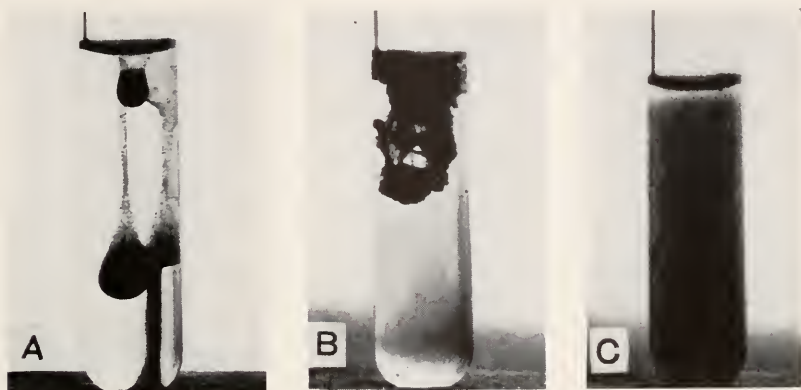


FIG. 1. Gross appearance of the egg-agglutination reaction. All photographs natural size.

(A) Five drops *Arbacia* eggs in 3 cc. of 5 per cent sperm extract. Note the drops of agglutinated eggs suspended from the surface of the solution.

(B) Ten drops of *Arbacia* eggs in 3 cc. of 5 per cent sperm extract. The tube was shaken once. The eggs have formed a large dense mass which floats at the surface.

(C) Ten drops of *Arbacia* eggs in 3 cc. heated sea water. The tube was shaken once after adding the eggs. Observe that the ova are homogeneously suspended and have begun to settle as shown by the clear area immediately below the surface.

The details of the egg agglutination reaction may be observed microscopically by making an egg suspension in Chinese ink which reveals the colorless jelly layer, or chorion, surrounding each ovum. By means of a capillary pipette, sperm extract is introduced into a drop of this mixture mounted on a slide beneath a raised coverslip and observed under high power. As the extract comes in contact with an egg, a faint black line of adhering ink particles immediately appears at the periphery of the jelly. This line rapidly increases in thickness until the chorion is encircled by a dense membranous structure which will be termed the "agglutination membrane."

The microscopic appearance of eggs treated with sperm extract is shown in Plate I, Figs. 2-5. A normal egg is surrounded by a wide zone of clear colorless jelly with poorly-defined margins (Fig. 2). On treating such an egg with sperm extract, a distinct agglutination membrane forms at the periphery of the jelly (Fig. 3), which gradually increases in density (Fig. 4). Particles of jelly freely suspended in the solution also agglutinate to form a dense interlacing network connecting adjacent eggs (Fig. 5).

With these changes the jelly becomes very adhesive, causing the ova to stick firmly together in clusters when the coverslip is moved about. Woodward (1915) states that the jelly of *Arbacia* eggs placed in "boiled sperm suspension" swells and becomes sticky so that the eggs adhere to each other and to the container. This observation was repeatedly confirmed during the present study with the exception that swelling of the jelly was never noted, either in boiled sperm suspensions or in sperm extracts. In some instances the chorion retains its original width but it generally shrinks markedly, as shown by a decrease in the space between agglutination membrane and cortex after prolonged exposure to the solution.

The egg-agglutinating substance affects only that portion of the jelly with which it comes in contact and incomplete agglutination membranes involving one-half or less of the circumference of the chorion appear on those eggs which have been only partially exposed to the extract.

In general, the density and rate of formation of the agglutination membranes are directly related to the concentration of extract. A 4 per cent sperm extract clots the eggs immediately into a solid mass surrounded by a thick unbroken line of agglutinated jelly; as the extract decreases in concentration, fewer eggs are involved, the reaction is slower, and the membranes are thinner until below about 0.5 per cent both gross and microscopic reactions become negative.

The egg-agglutination membranes grow tougher and more resistant to tension with time. After a few minutes exposure to a 2 per cent extract, the coverslip may be gently agitated without altering the circular shape of the membranes and the eggs move about freely in the unaffected central jelly. When greater tension is applied by rapidly agitating the coverslip, the membrane ruptures and the ovum may then be torn away leaving the membrane firmly anchored to the slide with its frayed edges waving about in the solution.

The egg-agglutination reaction is irreversible. Eggs which have once agglutinated remain firmly massed together in spite of transfer to sea water and eventually cytolysed *in situ*. Vigorous shaking increases

the size and density of the egg-clusters and leads to rapid destruction of the ova.

The jelly of *Arbacia* eggs binds the egg-agglutinating substance and removes it from the extract. This is illustrated by a typical experiment: 1 cc. shed eggs was added to 3 cc. of 20 per cent sperm extract and the agglutinated egg-mass was filtered off. The filtrate weakly agglutinated 1 cc. eggs, after which the solution was again filtered but this filtrate failed to agglutinate .5 cc. eggs. Three cc. of the same extract diluted with 2.5 cc. sea water as control strongly agglutinated eggs. The egg-agglutinating substance present in the 20 per cent extract has, therefore, been fixed by the jelly of 2 cc. eggs.

Sperm extracts not only act on the jelly but also on the cortex of *Arbacia* eggs. To study this, jellyless eggs were divided into two lots, one of which was fertilized with fresh sperm. Samples from each lot were fixed in Bouin's solution (without acetic acid) or 2 per cent osmic acid and then washed thoroughly in sea water. Jellyless eggs treated with sperm extract form clusters which are too minute to be reliably detected grossly so a microscopic method was used: a drop of jellyless eggs was mixed with a few drops of extract on a slide and a glass needle was moved through the mixture. The appearance of distinct egg-clusters represented a positive agglutination test.

Numerous experiments demonstrated that the cortex undergoes certain changes in sperm extracts causing the eggs to fuse so firmly together that bits of cytoplasm may be ripped out of the ovum by agitating the egg-clusters. In addition the fertilization membranes of fertilized jellyless eggs adhere to each other and may be pulled completely away from the zygote. These changes take place equally in unfertilized and fertilized jellyless eggs whether living or dead. The structure of the cortex, however, does not appear to be visibly altered by exposure to sperm extracts. Controls of the same eggs in heated sea water remained singly spaced in all cases, except for occasional small clusters caused by the use of acid in removing the jelly (Lillie, 1915a).

Identical experiments on normal eggs showed that the jelly of fertilized and unfertilized eggs, both living and dead, agglutinates in sperm extracts. Immature oocytes, with or without jelly, agglutinate as readily as mature ova.

To sum up: (1) The egg-agglutination reaction is not dependent on living protoplasm, since dead ova agglutinate as readily as living ones. (2) Immature, unfertilized, and fertilized eggs agglutinate equally, indicating that the developmental state of the ovum plays no part in the reaction. (3) Sperm extracts agglutinate jellyless eggs by directly affecting the cortex which becomes markedly adhesive.

One of the first questions to arise was whether the egg-agglutinating substance could be extracted from tissues or cells of *Arbacia* other than sperm. Extracts of *Arbacia* shells, lanterns, peristomes, ovaries, eggs, and coelomic fluid, prepared by heating 4 cc. of each tissue one minute in 10 cc. of sea water, were found to be without effect on ova. Extracts of immature testes or ripe testes washed free of sperm were likewise inert. The egg-agglutinating substance is thus highly tissue-specific and can be extracted solely from sperm. Moreover, sperm extracts act specifically on *Arbacia* eggs and will not agglutinate blood corpuscles, spermatozoa or any other cells of the male or female sea-urchin.

The egg-agglutination reaction is not confined to the gametes of *Arbacia* and a certain degree of cross-agglutination exists between eggs and sperm extracts of related and distant species. Preliminary experiments on the gametes of *Nereis* and *Echinarachnius* gave the following results: (1) *Echinarachnius* sperm extracts agglutinate ova of *Echinarachnius* and *Arbacia*. (2) *Nereis* sperm extracts agglutinate *Arbacia* eggs but do not agglutinate fertilized or unfertilized *Nereis* eggs. In *Nereis*, jelly extrusion occurs after fertilization, hence both fertilized and unfertilized ova were tested for agglutination. Just (1922) found that *Nereis* sperm boiled in sea water causes a small percentage of jelly formation, maturation, and differentiation without cleavage of unfertilized *Nereis* eggs. (3) Sperm extracts of *Arbacia* agglutinate *Echinarachnius* eggs. Such extracts will not agglutinate *Nereis* eggs but cause jelly extrusion and maturation (without cleavage) of unfertilized *Nereis* eggs.

The chemical specificity of the egg-agglutination reaction is less distinct than the biological. *Arbacia* eggs are agglutinated by certain salts, foreign sera (Robertson, 1912), acids and alkalis.

PHYSICAL AND CHEMICAL PROPERTIES OF THE EGG-AGGLUTINATING SUBSTANCE

These experiments were devised to study certain general physical and chemical properties of the egg-agglutinating substance and do not represent exact quantitative studies. Each experiment was repeated several times with different materials and the results were consistent in each case. The egg-agglutination tests were standardized by adding three drops of fresh eggs to 2 cc. of sperm extract in a test tube and shaking the tube lightly. In certain experiments the agglutinating strength of the extracts was rated as follows:

- 0 = No agglutination. All eggs homogeneously suspended.
1 = Few thin shreds of agglutinated eggs with the majority freely suspended.
2 = One or more loose clusters of agglutinated eggs with some ova freely suspended.
3 = Single dense clot of agglutinated eggs with no eggs free in the solution (maximum).

Appearance, Specific Gravity, and pH of Sperm Extracts

Extracts of clean shed sperm are colorless while extracts of testis sperm have a light purple tint ("purple X" of Glaser, 1914). Dilute sperm extracts are clear; those of high concentration (40 per cent) are faintly opalescent. The specific gravity of a 50 per cent extract is 1022, which about equals that of sea water (av. sp. gr. = 1028). The pH of a 60 per cent sperm extract in sea water, measured with the Coleman glass electrode, is 7.74; that of a 1 per cent extract is 8.02. The slight relative acidity of the 60 per cent extract probably results from CO₂ liberated by the thick sperm suspension prior to extraction. These values approximate the pH of sea water at Woods Hole (av. pH = 8.00).

Relation of Temperature to the Extraction and Stability of the Egg-agglutinating Substance

To determine whether the egg-agglutinating substance can be extracted from sperm at low temperatures, 10 cc. sea water was heated in a waterbath to a given temperature, a measured amount of mixed shed sperm was stirred into the sea water and kept for one minute at the desired temperature, after which the solution was filtered. Under these conditions it was found that sea water below about 57° C. will not extract the egg-agglutinating substance. This can be extracted in small amounts from a 20 per cent sperm suspension at 57° and from a 1 per cent suspension at 68° C. Higher temperatures extract greater quantities of the substance from sperm. There appears to be an inverse relation between sperm concentration and the critical temperature of extraction.

The rate of loss of the egg-agglutinating substance on standing is a function of the temperature of the extract: 50 cc. of a 10 per cent sperm extract was divided into two equal parts, one of which was aged at room temperature (26° C.) and the other in an icebox (7° C). The results of tests on each solution at various times are given in Table I.

The agglutinating strength of the extract kept at 7° C. remained constant for twenty-five hours and then gradually diminished and dis-

appeared after forty-five hours. The same extract at 26° C. lost its activity in less than thirteen hours. In a similar experiment, a 1 per cent extract lost its agglutinating power in less than twelve hours at 26° C. and in twenty hours at 7° C. showing that the egg-agglutinating substance disappears more rapidly from dilute extracts. We may conclude that this substance is preserved best at low temperatures.

The egg-agglutinating substance is highly heat-resistant and only slowly destroyed by boiling: 25 cc. of a 20 per cent sperm extract was boiled for several hours in a beaker, the volume being kept constant by addition of distilled water. The agglutinating strength of the extract remained high (3) for four hours, then gradually decreased, and dropped to zero after five and one-half hours of boiling.

TABLE I

Egg-agglutinating strength of a 10 per cent sperm extract aged at 7° C. and 26° C. (3 = maximum)

Age of Extract <i>hours</i>	Temperature	
	7° C.	26° C.
0.....	3	3
13.....	3	0
25.....	3	—
38.....	2	—
41.....	1	—
45.....	0	—

Dialysis and Filtration

Thirty cc. of a 30 per cent sperm extract was divided into three equal parts (*A*, *B*, and *C*), each of which was placed in a Thomas Diffusion Shell (No. 4471). Extract *A* was dialysed against running sea water in a beaker, *B* was suspended in a cylinder containing 10 cc. sea water, and *C* was kept as a control. After twelve hours each extract was tested for its activity. If dialysis had occurred, one would expect a decrease in the concentration of the egg-agglutinating substance in extracts *A* and *B* and the appearance of this substance in the dialysate of extract *B*. By dilution of each solution with sea water, however, it was found that the concentration of the substance in *A* and *B* exactly equalled that of the control, *C*. The dialysate of *B* was totally inert. Numerous similar experiments proved conclusively that the egg-agglutinating substance is non-dialysable. Also it will not pass through a Berkefeld filter regardless of the concentration of sperm extract. These facts suggest that it is a colloidal substance of large molecular size.

Source of the Egg-agglutinating Substance

The sperm heads of *Arbacia* are composed chiefly of nucleic acids and "Arbacin," a substance having properties in common with both histones and protamines (Mathews, 1897). In view of the relatively minute amounts of lipids which are confined largely to the sperm tails, it seemed likely that the egg-agglutinating substance might be a protein or protein derivative. Sperm extracts were found to be negative to the following tests for proteins: Millon's, Adamkiewicz, biuret, xanthoproteic, and ninhydrin. No precipitate forms on adding saturated $(\text{NH}_4)_2\text{SO}_4$ or 70 per cent alcohol and no reduction occurs with Benedict's solution or bismuth subnitrate. Hence if proteins or carbohydrates are present in sperm extracts, their concentration is too minute to be detected by ordinary chemical tests.

The lipids of sperm may be readily separated from the proteins by alcohol-ether extraction. The procedure used was adapted after Bloor's method for extraction of total lipids from blood serum (Peters and Van Slyke, 1932, p. 495). Ten cc. of mixed shed sperm was shaken in 150 cc. alcohol-ether mixture (three parts 95 per cent alcohol plus one part ethyl ether) and the mixture boiled by immersion in a waterbath. The solution was made up to 200 cc. with alcohol-ether, cooled, and filtered through fat-free filter paper. The rubbery pink protein residue was washed thoroughly in sea water, heated one minute in 10 cc. sea water and filtered. The filtrate strongly agglutinated eggs.

The honey-colored lipid extract was evaporated to dryness on a waterbath, the residue was dissolved in 100 cc. petroleum ether, filtered, and re-evaporated to dryness. This procedure yielded a small quantity of yellow waxy material which was shaken in 10 cc. sea water, heated one minute, and filtered. The filtrate had no effect on eggs.

To determine whether heat was the factor responsible for liberating the egg-agglutinating substance in the above procedure, shed sperm was shaken in cold alcohol-ether solution, after which the protein precipitate was washed in sea water, shaken vigorously in 10 cc. sea water, and filtered. The filtrate markedly agglutinated eggs proving that heat is not essential for extraction.

To sum up, the egg-agglutinating substance is derived from the protein residue (head) of the spermatozoön and is not found in the lipid fraction.

Chemical Composition of the Extraction Medium

Sperm extracts made in tap or distilled water will not agglutinate eggs and if the residue from a sperm suspension, which has been heated

one minute in sea water, is reheated in distilled water, the extract is likewise inert. By substituting sea water for distilled water, in the above procedures, the egg-agglutinating substance is readily obtained. These facts suggested that certain salts or ions, present in sea water, are essential for the extraction of the substance. A thorough study of this problem requires chemical analyses of various extraction media and is beyond the scope of this investigation which is concerned with reactions occurring in sea water. The experiments cited below, therefore, represent preliminary studies:

Ten per cent sperm suspensions were made up in each of the following solutions and extracted by heating one minute and filtering:

1. Van't Hoff artificial sea water solutions each of which lacked a single salt present in normal sea water: CaCl_2 , MgSO_4 , MgCl_2 , KCl , and NaCl .

2. Pure solutions of each of the above salts in distilled water.

3. Pure solutions of Mg , Ca , K , and Na acetate in distilled water. The reagents were all made up isosmotic with sea water and the pH varied from about 7.2-9.0. (The viscosity, ionic concentration, and molarity could not be controlled.) None of the solutions visibly injured ova or sperm, or agglutinated eggs.

It was found that an active sperm extract agglutinated eggs washed in any of the above media to the same degree as eggs washed in sea water. Shed eggs in sea water were therefore used in the egg-agglutination tests.

From the results, listed in Table II, the following conclusions may be drawn:

- (1) Sea water lacking magnesium (MgCl_2 , MgSO_4 or both) will not extract the egg-agglutinating substance from sperm whereas pure solutions of MgCl_2 or MgSO_4 will. Magnesium is thus essential to the extraction process.

- (2) Sea water lacking calcium gave variable results; out of 6 tests, 3 were weakly positive and 3 negative. A pure solution of CaCl_2 readily extracts the egg-agglutinating substance. Calcium, therefore, appears to be necessary in sea water for the extraction of this substance.

- (3) Sperm extracts made in sea water which lacks either NaCl or KCl agglutinate eggs. Extracts made in pure NaCl or KCl solutions have no effect on eggs. These salts evidently play no part in the extraction of the egg-agglutinating substance in sea water.

- (4) If acetate ions are substituted for chloride ions in pure solutions of the salts of sea water, it is found that Mg and Ca acetate will extract the egg-agglutinating substance from sperm but Na and K

acetate will not. The egg-agglutination reaction is therefore not dependent upon the presence of any particular anions.

To sum up, the bivalent cations, magnesium and calcium, are the only ions of *sea water* which are necessary for the extraction of the egg-agglutinating substance from sperm. Both of these ions must be present in sea water but each will act alone when present in sufficient concentration in distilled water.

Extraction of Sperm in Acid and Alkaline Sea Water

In these experiments .10 N HCl and .10 N NaOH were diluted with varying quantities of sea water and the solutions used in preparing 5 per cent sperm extracts.

TABLE II

The agglutination of eggs by 10 per cent sperm extracts prepared in various isosmotic salt solutions. Three drops of eggs added to 2 cc. of each extract. + = positive tests. 0 = negative tests. All solutions prepared by Marine Biological Laboratory Chemical Room.

Extraction Solution	Egg-agglutination
* Artificial Sea Water (Van't Hoff)	+
* Ca-free Sea Water	+
* Mg-free Sea Water	0
* MgSO ₄ -free Sea Water	0
MgCl ₂ -free Sea Water	0
KCl-free Sea Water	+
* NaCl-free Sea Water	+
.34 M CaCl ₂	+
* .37 M MgCl ₂	+
* MgSO ₄ ·7H ₂ O	+
.53 M KCl	0
.52 M NaCl	0
Ca Acetate	+
Mg Acetate	+
K Acetate	0
Na Acetate	0

* Indicates extracts on which fertilizin-inactivation tests were run with results parallel to those for egg-agglutination tests.

Lillie (1915a) states that acid sea water heavily agglutinates and later cytolyses *Arbacia* eggs. The present study shows that when sperm is extracted in acid sea water of above .003 N HCl content, the agglutinating effect of the acid hides the action of the egg-agglutinating substance. These substances, however, have different actions since HCl agglutinates and later cytolyses eggs whereas the egg-agglutinating substance agglutinates but does not kill the ova. Sperm extracts in sea water of or below .003 N HCl content agglutinate eggs. Sea water of

this degree of acidity has no effect, which proves that the agglutination is here caused by the egg-agglutinating substance alone.

Haas (1916), Irving (1926), and Kapp (1928) have shown that the addition of .10 N NaOH to sea water precipitates most of the magnesium and some calcium as hydroxides. In the present study it was found that alkaline sea water containing more than .01 N NaOH loosely flocculates *Arbacia* eggs, probably due to precipitation of calcium and magnesium hydroxide in the egg jelly. This precipitate is removed when alkaline sea water is heated and filtered (as in preparing sperm extracts) and eggs added to the filtrate remain singly spaced. It is thus possible to determine the highest concentration of alkali in sea water which will permit the extraction of the egg-agglutinating substance from sperm. This was found to be about .012 N NaOH sea water; above this degree of alkalinity no agglutination takes place; below it the extracts agglutinate eggs. The previous section has shown that sea water lacking Mg or Ca will not extract the egg-agglutinating substance from sperm. According to Irving (1926), .017 molar NaOH precipitates 19 per cent of the total Ca and 16 per cent of the total Mg from sea water. A possible explanation for the action of alkaline sea water in extracting sperm is that sufficient Mg and Ca are removed from sea water by concentrations of alkali above .012 N NaOH to render it inert as an extracting medium. Below this degree of alkalinity, however, enough ionized Mg and Ca are present to allow extraction of the egg-agglutinating substance.

The pH of the solutions varied from 3.2 (.003 N HCl in sea water) to 9.5 (.012 N NaOH in sea water). Since sperm extracts made in either of these solutions agglutinate eggs, the egg-agglutination reaction takes place over a wide range of hydrogen-ion concentrations.

THE INACTIVATION OF FERTILIZIN BY SPERM EXTRACTS

As is well known from the classical studies of F. R. Lillie (1913-1919), mature unfertilized eggs of *Arbacia* secrete a substance into sea water which reversibly agglutinates sperm of the same species. Lillie named this substance "fertilizin" and concluded that it functions in fertilization as an essential chemical link in the union of egg and spermatozoön. For further details concerning the fertilizin theory of fertilization see the recent review of Just (1930).

Since fertilizin is present in high concentration in the jelly of *Arbacia* eggs, it seemed probable that sperm extracts might react in some way with this substance, which proved to be correct. When sperm extracts are mixed with egg-water, the latter loses its capacity to agglutinate sperm; some substance in the extract has neutralized the

fertilizin. For description purposes we will call this "fertilizin-inactivation" and the hypothetical substance responsible for it the "fertilizin-inactivator."

To study the relation of fertilizin-inactivation to the concentration of sperm extract, 2 cc. of successive half-dilutions of an 8 per cent extract was placed in a series of test tubes and 2 cc. egg-water of known fertilizin content was added to each tube. This procedure was repeated with egg-waters of descending fertilizin content, the concentration of sperm extracts remaining constant. The highest dilution of egg-water was mixed with an equal quantity of heated sea water as a control. A drop of each mixture was tested for fertilizin by its ability to agglutinate a drop of fresh 1 per cent sperm suspension; absence of sperm agglutination constituted a positive fertilizin inactivation test; agglutination of sperm, indicating the presence of free fertilizin in the mixture, was considered a negative fertilizin-inactivation test.

In Table III, which summarizes the results of three experiments, a change from + to 0 in each horizontal row represents the end-point at which the extract in the corresponding vertical column becomes too dilute to inactivate all of the fertilizin present. For example, 400 fertilizin units are inactivated by a 4 per cent but not by a 2 per cent sperm extract. In each experiment at least three determinations were made at each of these end-points with different sperm suspensions as indicator in order to reduce error caused by variation in materials. Note (Table III) that an 8 per cent extract inactivates 800 fertilizin units whereas a .06 per cent extract only inactivates 10; a .1 per cent extract inactivates 20 but not 40 fertilizin units. The controls agglutinated sperm in all instances. Sperm extracts thus inactivate fertilizin in roughly quantitative proportions, the capacity for inactivation varying directly with the concentration of extract.

Certain incidental phenomena were noted during the course of this experiment. When a drop of concentrated sperm extract plus egg-water is added to a drop of spermatozoa, the latter form a dense, highly active ring around the introduced drop but sperm agglutination does not occur. Sperm extracts therefore react only with fertilizin without altering the "sperm-activating and aggregating" substances of egg-water, which confirms Lillie's statement (1914) that these substances are distinct from fertilizin. Furthermore, it was noted that on shaking concentrated sperm extract plus egg-water mixtures, a light purple, flocculent precipitate appears and the egg-water, which previously was purple, immediately becomes colorless. The precipitate floats at the surface of the solution and is probably formed by agglutination of sub-microscopic jelly particles present in the egg-water.

It may be pointed out here that fertilizin does not appear to be essential to the egg-agglutination reaction, since fertilized jellyless eggs washed free of fertilizin agglutinate as readily as unfertilized jellyless eggs which secrete large quantities of this substance.

TABLE III

Combined results of three experiments on the inactivation of fertilizin by sperm extracts. Upper horizontal column gives concentration of sperm extracts; vertical column gives concentration of egg-water in units of fertilizin. For each test, 2 cc. egg-water was added to 2 cc. extract and the mixture tested for its ability to agglutinate a 1 per cent sperm suspension. + = positive tests (no sperm agglutination); 0 = negative test (sperm agglutination).

Concentration of Sperm Extract:	8%	4%	2%	1%	0.5%	0.2%	0.1%	0.06%	0.03%
Fertilizin Units	Fertilizin-Inactivation Tests								
800	+	+	0	0	0				
	+	+	0	0	0				
	+	+	0	0	0				
400	+	+	0	0	0	0			
		+	0	0	0				
200	+	+	+	0	0	0			
		+	+	+	0	0			
			+	+	0	0			
100		+	+	+	0	0			
		+	+	+	0	0			
80			+	+	+	0	0		
			+	+	+	0	0		
			+	+	0	0	0		
40			+	+	+	0	0		
			+	+	+	+	0		
				+	+	0	0		
20				+	+	+	+	0	
				+	+	+	+	0	
					+	+			
10					+	+	+	+	+
					+	+	+	+	0

PROPERTIES OF THE FERTILIZIN INACTIVATOR

We have described two effects of sperm extracts, one on eggs and the other on fertilizin. To determine whether these effects are related

to each other, simultaneous tests for the egg-agglutinating substance and fertilizin-inactivator were run on sperm extracts. The fertilizin-inactivation tests represent a part of the previously described experiments dealing with the properties of the egg-agglutinating substance and the results of the former will, therefore, be briefly summarized without repeating experimental details. The fertilizin-inactivation tests were standardized by adding .3 cc. sperm extract to .3 cc. egg-water of known fertilizin content and testing the mixture for agglutination on a fresh 1 per cent sperm suspension. The inactivating strength of a sperm extract was considered equal to the maximum number of fertilizin units which it neutralized.

The fertilizin-inactivator is markedly tissue-specific and cannot be extracted from any tissue or cells of *Arbacia* except sperm. It has long been known that blood and tissue extracts of *Arbacia* will not combine with fertilizin but that extracts of washed unfertilized eggs contain a substance, "anti-fertilizin," which neutralizes minute amounts of fertilizin (Lillie, 1914). Because anti-fertilizin combines only with fertilizin without affecting the sperm-aggregating and activating factors of egg-water, it acts like the fertilizin-inactivator, but the latter is much more potent in neutralizing fertilizin.

TABLE IV

Fertilizin-inactivating strength of a 10 per cent sperm extract aged at 7° C. and 26° C. Inactivating strength given as maximum number of fertilizin units inactivated by extract.

Age of Extract hours	Temperature	
	7° C.	26° C.
0.....	400	400
13.....	400	50
25.....	200	—
38.....	100	—
41.....	50	—
45.....	25	—

Low temperatures preserve the fertilizin-inactivator as shown in Table IV, which gives the data from a single experiment. After thirteen hours at 26° C. the inactivating strength of a 10 per cent extract dropped from 400 to 50; after twenty-five hours at 7° C. the inactivating strength fell from 400 to 200 and then gradually decreased to 25 in forty-five hours.

The fertilizin-inactivator is quite thermostable and is gradually destroyed by boiling. The inactivating strength of a 20 per cent sperm

extract fell from 400 to 50 after two and one-half hours at 100° C. and remained at 50 after five and one-half hours of boiling.

It can be obtained from the protein residue but not from the lipid fraction when sperm is extracted with hot alcohol-ether and it is extractable from sperm shaken in cold alcohol-ether. It will not dialyze or pass through a Berkfeld filter and is not present in sperm extracts made in tap or distilled water. In the previously described experiments with artificial sea water solutions, fertilizin-inactivation tests were run on a few of the extracts (marked with a (*) in Table II) and the results paralleled the egg-agglutination tests in each case.

Because the egg-agglutinating substance and fertilizin-inactivator in general showed parallel behavior in all experiments they will be referred to from now on collectively as the "active principles" of sperm extracts.

SECRETION OF ACTIVE PRINCIPLES AND THEIR RELATION TO THE FERTILIZING POWER OF SPERM

Filtrates of fresh or old sperm suspensions which have been passed through Whatman filter paper and the supernatant fluid of centrifuged sperm suspensions will not react with fertilizin or eggs. The active principles are found only in sperm *extracts* and are not secreted by sperm into water.

It was found that extracts of sperm which have been aged until dead are inert. The fact that the active principles can only be extracted from fresh sperm suggested that they might be related to the fertilizing power of sperm. Experiments were therefore undertaken to determine whether the loss of fertilizing power of aging sperm suspensions bears any relation to the loss of active principles from extracts of these suspensions. Sperm suspensions of various concentrations (8 per cent, 4 per cent, 2 per cent, and 1 per cent) were prepared by adding measured amounts of mixed shed sperm to 80 cc. sea water in a series of Erlenmeyer flasks. The sea water was sterilized in an autoclave to lessen bacterial growth which shortens the life of sperm (Cohn, 1918). The flasks were closed with sterile corks and aged at room temperature (26°–28° C.) for three days. At intervals 4 cc. of each suspension was heated thirty seconds, filtered, and the extract divided into two equal parts which were tested for the active principles.

In the fertilizin-inactivation tests, in order to insure complete inactivation of fertilizin by each extract at the start of the experiment, descending concentrations of fertilizin were added; 200 fertilizin units were added to the 8 per cent, 100 to the 4 per cent, 50 to the 2 per cent and 25 to the 1 per cent sperm extract.

Simultaneously with these tests, the fertilizing power of the sperm was measured by adding one drop from each sperm suspension to three drops of fresh eggs in 10 cc. sea water and counting the percentage of fertilized eggs in each dish two hours later.

TABLE V

The relation between loss of fertilizing power of aging sperm suspensions and loss of active principles from extracts of these suspensions. Horizontal rows give simultaneous tests made at intervals recorded at left. *A* = egg-agglutination tests. *B* = fertilizin inactivation tests; the figures in brackets are the number of fertilizin units added to each extract. *C* = fertilizing power of sperm suspensions expressed as the percentage of eggs fertilized by each suspension. + = positive tests; 0 = negative tests.

Age of Sperm Suspensions	<i>A</i>				<i>B</i>				<i>C</i>			
	Sperm Extracts				Sperm Extracts				Sperm Suspensions			
	Egg-agglutination Tests				Fertilizin-inactivation Tests				Percentage of Eggs Fertilized			
					(200)	(100)	(50)	(25)				
					8%	4%	2%	1%				
hours	8%	4%	2%	1%	8%	4%	2%	1%	8%	4%	2%	1%
24.....	+	+	+	0	+	+	+	+	100	100	100	100
30.....	+	+	+	+	+	+	+	+	100	99	100	89
36.....	+	+	+	0	0	+	+	+	100	100	100	94
49.....	+	+	0	0	+	+	0	0	100	93	12	11
56.....	+	+	0	0	+	+	0	0	100	13	0	3
72.....	0	0	0	0	0	0	0	0	26	0	0	0

The data are recorded in Table V. Column *A* lists the egg-agglutination tests, column *B* the fertilizin-inactivation tests, and column *C* the fertilizing power of each suspension. Each horizontal row thus records comparable tests at various times. For the first thirty-six hours the active principles are present, in general, in all extracts except for the 1 per cent which has lost its capacity to agglutinate eggs. Throughout this period the sperm suspensions fertilize 89–100 per cent of the eggs. After forty-nine hours, both egg-agglutination and fertilizin-inactivation tests are negative in the 2 per cent and 1 per cent extracts and at this time the fertilizing power of the 2 per cent and 1 per cent sperm suspensions has dropped to 12 per cent and 11 per cent respectively. The active principles have disappeared in all extracts after seventy-two hours and the fertilizing power of each suspension has dropped to zero except for the 8 per cent suspension which fertilizes

26 per cent of the eggs. At the close of the experiment living sperm were present in each suspension (except the 1 per cent) as shown by clear cytoplasm of the sperm heads and slight motility.

Many sources of error are present in an experiment of this sort which cannot be adequately controlled. The necessity for testing the same solutions for three days introduces unavoidable contamination and the condition of the eggs and sperm used for testing varies widely in different urchins from day to day (Goldforb, 1929). Nevertheless, it is clear that the loss of fertilizing power of aging sperm suspensions parallels the loss of active principles from extracts of these suspensions.

THE EFFECT OF SPERM EXTRACTS ON FERTILIZATION AND DEVELOPMENT

Effect of Sperm Extracts on Both Gametes

In an extensive series of experiments, normal and jellyless eggs were fertilized in sperm extracts (.1 per cent to 8 per cent), transferred to sea water after exposure to the extracts for from thirty seconds to three hours, and at various times counts were made of the percentage of eggs developing. Controls consisted of the same eggs fertilized in heated sea water. Fertilization and development in extracts below about 2 per cent concentration equals that of the controls but above this concentration marked inhibition occurs. The majority of ova remain unfertilized and no fertilization membranes appear despite the presence of numerous active sperm at the cortex. Those eggs which are activated usually cleave normally but cease developing at or before the non-motile blastula stage if allowed to remain in the extracts. When the embryos are transferred to sea water they develop into normal plutei. These effects occur in both jellyless and normal eggs but the inhibition is greater in the latter, probably due to agglutinated jelly which physically prevents escape of the blastulae from the egg-masses.

In these experiments both germ-cells were simultaneously exposed to sperm extracts and the block to fertilization could be due to the action of extracts on the ovum alone, spermatozoön alone or on both gametes. We will now consider the action of extracts on each gamete.

Effect of Sperm Extracts on the Fertilizing Power of Sperm

Sperm suspensions were made in sperm extracts and fresh eggs fertilized with these mixtures: 1 per cent sperm suspensions were prepared in .5 per cent to 8 per cent extracts and after exposure of sperm to the extracts for various times, one drop from each suspension was used to fertilize three drops of fresh eggs in 20 cc. sea water. The per-

centage of fertilized eggs was counted two hours after insemination. A 1 per cent sperm suspension in boiled sea water served as control.

TABLE VI

The inhibiting effect of sperm extracts on the fertilizing power of sperm. One per cent sperm suspensions prepared in sperm extracts of various concentrations. Figures give percentage of fresh eggs fertilized by one drop from each suspension after exposure of sperm to the sperm extracts for times given at left. Control = 1 per cent sperm suspension in boiled sea water.

Concentration of Sperm Extracts	8%	4%	1%	0.5%	Control
Time Sperm Exposed to Sperm Extracts	Percentage Eggs Fertilized				
5 seconds	18	1	68	—	
15 seconds	3	2	20	92	
45 seconds	8	3	17	100	
60 seconds	7	5	18	92	
15 minutes	3	3	32	12	
30 minutes	3	1	5	4	99
60 minutes	1	1	1	1	99

From Table VI, which gives the result of one experiment, it is seen that the percentage of eggs fertilized by sperm suspensions in extracts of above 1 per cent drops greatly after fifteen seconds at which time the 4 per cent suspension fertilizes only 2 per cent of the ova. After fifteen minutes exposure, the fertilizing power of the .5 per cent suspension has dropped from 92 per cent to 12 per cent; after thirty minutes the percentage of eggs fertilized by each suspension has fallen almost to zero. The controls fertilized 99 per cent of the ova after one hour. Numerous motile sperm were seen at the surface of the ova but fertilization membranes did not appear. Sperm extracts, then, block fertilization by a rapid inhibiting effect on sperm which lose their fertilizing power but retain motility. The loss of fertilizing power is directly proportional to both the concentration of extract and the time of exposure.

Effect of Sperm Extracts on the Fertilizability of Eggs

These experiments were run on jellyless eggs in order to permit direct contact between cortex and sperm extract and to eliminate any mechanical interference with fertilization due to agglutination of the egg jelly. Twenty drops of jellyless eggs from a single female were placed in 10 cc. sperm extracts (.1 per cent to 8 per cent) and at intervals ten

drops of eggs were transferred from each extract to 10 cc. sea water. The ova were fertilized immediately after transfer by two drops of a 1 per cent sperm suspension (made up fresh at the start of the experiment and used throughout) and the percentage of eggs developing was counted two hours later. Twenty drops of the same eggs in boiled sea water were treated in the same manner and served as control.

TABLE VII

The inhibiting effect of sperm extracts on the fertilizability of eggs. Jellyless eggs added to sperm extracts of various concentrations, transferred to fresh sea water, and immediately inseminated with fresh sperm. Figures give percentage eggs fertilized after exposure to the extracts for the times given at left. Control = same eggs in boiled sea water.

Concentration of Sperm Extracts	8%	2%	0.5%	0.1%	Control
Time Jellyless Eggs Exposed to Sperm Extracts	Percentage Eggs Fertilized				
<i>minutes</i>					
15.....	20	57	91	91	
30.....	24	45	90	98	
60.....	15	17	89	96	100
90.....	21	21	87	97	
120.....	2	8	66	93	92

The data of a typical experiment are given in Table VII. After two hours exposure to extracts of above 2 per cent, less than 10 per cent of the eggs are fertilized. At this time less inhibition occurs in the eggs exposed to the .5 per cent extract since 66 per cent are fertilized. The .1 per cent extract equals the controls (91-98 per cent fertilized). We may conclude that the inhibiting effect of sperm extracts on the fertilizability of eggs is due to an effect on the cortex and is directly related to the concentration of extract and length of exposure.²

Effect of Sperm Extracts on Fertilized Eggs and Embryos

We have seen that fertilization and development of eggs fertilized in sperm extracts are inhibited when the gametes are exposed to extracts

² Glaser (1914) stated that eggs which have been treated for two hours with "boiled sperm infusion" remain unfertilized when heavily inseminated. He believed this inhibition to be due to "purple X" since boiled sperm infusions from which the color had been removed did not block fertilization. Woodward (1915) recorded similar results. In the present study it was found that the presence of coloring matter did not appear to influence in any way the inhibiting action of sperm extracts on the fertilizability of eggs.

during their union in fertilization. The inhibiting effect of sperm extracts also occurs *before* the union of the germ-cells, involving egg and sperm individually. We will now consider the action of sperm extracts on eggs *after* normal fertilization.

Jellyless and normal eggs were inseminated in sea water, transferred to sperm extracts (8 per cent to .1 per cent) ten minutes after fertilization, and some of the eggs were transferred back to sea water after exposure to the extracts for from thirty seconds to one hour. Controls were the same eggs in sea water. The following observations were made:

Three hours after fertilization—Normal cleavage in all extracts equal to that of controls.

Seven hours after fertilization—Development of eggs in extracts of above 2 per cent concentration stopped at non-motile blastula stage. Healthy motile blastulae found in remaining extracts and controls.

Twenty-three hours after fertilization—Embryos in 8 per cent extract have cytolysed at the blastula stage. Rare plutei in the 2 per cent extract with majority cytolysed at blastula stage. Healthy plutei found in remaining extracts and controls.

Throughout the series the ova transferred to sea water developed into normal plutei, indicating that the inhibitory effect of sperm extracts is reversible. In the concentrated extracts normal eggs were inhibited to a slightly greater degree than jellyless eggs due to agglutinated egg-masses preventing escape of the blastulae.

When a drop of sperm extract is added to a drop of free-swimming plutei in Chinese ink, the embryos are paralysed at once on entering the extract and fall to the bottom. Peristaltic movements of the gut and feeble ciliary motion are the only indications that the embryos are alive. Under high power the cilia are stuck together by their tips and the plutei are surrounded by clumps of ink particles enmeshed in the agglutinated cilia. Due to the increased stickiness of the cilia, the plutei adhere together in large clusters when a glass needle is moved through the solution. This phenomenon occurs in plutei which have developed from completely jellyless eggs and so is not the result of agglutination of particles of egg jelly by the sperm extract.

In attempting to explain these findings it must be realized that extracts of whole sperm undoubtedly contain numerous unknown substances but the action of the egg-agglutinating substance accounts for many of the above facts. We have seen that eggs fertilized in sperm extracts or placed in extracts after fertilization in sea water proceed

normally to the non-motile blastula stage and then abruptly stop developing. It is likely that the cilia, which are pushed forth from the surface of the blastula at this time, are immediately agglutinated by the extract with the result that the blastulae are unable to break out of the fertilization membranes and die *in situ*. When embryos are transferred to sea water from the extract, the cilia are able to function, motility follows, and development continues normally. Paralysis of motile embryos by sperm extracts is probably also caused by agglutination of the cilia.

PARTHENOGENESIS WITH SPERM EXTRACTS

Repeated attempts were made to initiate development in unfertilized *Arbacia* eggs by exposing them to sperm extracts. Both normal and jellyless eggs were immersed in extracts of various concentrations (.5 per cent to 100 per cent) for from five seconds to twenty-four hours and then transferred to sea water. In other experiments eggs were treated with mixtures of egg-water and sperm extracts, extracts from which the active principles had been removed by boiling or aging, dialysed extracts, etc. The results were entirely negative in all instances. It is clear that immersion of eggs in sperm extracts is not sufficient stimulus for development. Perhaps local application of sperm extracts may be effective.

DISCUSSION

We have seen that there are two major effects of sperm extracts, one upon the surface of the egg and the other a reaction with fertilizin, which paralleled each other under various experimental conditions. It is not possible to determine whether these effects represent the activity of two distinct substances or two properties of a single substance since the indicators for each are different; eggs for the egg-agglutinating substance and fertilizin for the fertilizin-inactivator. It will be assumed that a single "active substance" is present in sperm extracts which reacts with eggs and fertilizin.

The question of chief importance relating to this study is whether the active substance functions in fertilization. This substance is extractable only from sperm, agglutinates *Arbacia* eggs and embryos but has no effect on *Arbacia* sperm or blood corpuscles, and combines specifically with fertilizin which is believed to play an important part in fertilization. This marked degree of tissue specificity between eggs, fertilizin, and the active substance suggests that the latter is related to fertilization.

Because sperm loses its fertilizing power before loss of motility, Lillie (1915*b*) postulated that sperm contains a "fertilizing substance"

which combines with fertilizin and thereby activates the ovum. Such a substance, if present in sperm extracts, must theoretically (1) combine with fertilizin and neutralize its capacity to agglutinate sperm, (2) be lost with the fertilizing power of sperm, and (3) activate unfertilized eggs. Since sperm extracts inactivate fertilizin in quantitative proportions and loss of fertilizing power of sperm suspensions parallels loss of the active substance from extracts of these suspensions, two of the above criteria are fulfilled. On the other hand, sperm extracts will not activate eggs, which would tend to disprove the similarity of the fertilizing and active substances. It is possible that the active substance is non-diffusible and hence cannot permeate the cortical membrane, local application is necessary to cause activation, or a penetrating force (sperm) is essential to carry the fertilizing substance into the cytoplasm. Fertilization might then result from injecting sperm extracts into the cortex or increasing the permeability of the vitelline membrane. The block to fertilization caused by sperm extracts is additional evidence against the identity of the active and fertilizing substances. Assuming that fertilizin is necessary for activation, the inhibiting effect of sperm extracts on the fertilizability of eggs may be due to the neutralization of fertilizin at the egg surface by the active substance of sperm extracts. Possibly agglutination of the peripheral layer of the cortex physically prevents sperm penetration.

Lillie (1919, p. 219) suggests that there is a high degree of chemical specificity in the union of egg and sperm and Just (1930, p. 331), defending Lillie's use of immunological terms in his fertilizin theory, states, ". . . The biology of fertilization has more points in common with immune reactions than with any other biological phenomenon." The active substance of sperm extracts resembles bacterial agglutinins in certain respects. The following properties of bacterial agglutinins are taken from Wells (1929) and Kolle and Hetsch (1935): (1) Agglutinins disintegrate in sera when preserved, are non-dialysable, pass only incompletely through Chamberland filters, and are not destroyed by extracting serum with lipid solvents. (2) Electrolytes are necessary for agglutination reactions, which will not occur in distilled water. The cation and not the anion of the added salt is of importance. The reactions take place over a wide range of hydrogen-ion concentration (pH 3.7-9.0). (3) The specificity of agglutination reactions is less marked than that of other immune reactions due to the complex structure of cells which permits similar proteins, carbohydrates and lipids to occur in cells of different species; different species of bacteria will thus react with a single agglutinin. (4) Dead bacteria agglutinate as readily as living ones and the agglutination of living bacteria does not kill them. In each

of the above properties there is a remarkable similarity between the agglutination of bacteria by sera and the agglutination of eggs by sperm extracts. Certain differences are also found; thus all known agglutinins are destroyed at or below 70° C. whereas the egg-agglutinating substance resists boiling for hours.

One of the theories concerning the mechanism of agglutination is that the agglutinin reduces the electronegative charge which in part keeps bacteria (or other cells) in suspension, thereby rendering them more susceptible to the precipitating action of salts. We have pointed out that egg-agglutination occurs only in the presence of certain cations (Mg or Ca) and according to Heilburnn (1923), the surface of *Arbacia* eggs is negatively charged. Possibly the active substance of sperm extracts reduces this negative charge at the surface of the ovum and permits the cations of the electrolyte to precipitate the eggs. The agglutination membrane would then form by precipitation of dissolved colloids at the periphery of the jelly, which has been "sensitized" to the precipitating action of salts by the active substance.

The active substance appears to be related to fertilizin in that both substances are non-dialyzable, thermostable, colloidal, and do not give the usual protein tests. It is likely that the active substance and fertilizin belong to a class of chemical compounds related to, if not identical with, bacterial agglutinins.

In conclusion, the remarkable tissue specificity of the active substance, its ability to combine selectively with fertilizin, its agglutinating action on eggs and embryos, its relation to the fertilizing power of sperm, and finally, the analogies between immune reactions, fertilization, and the properties of the active substance, all suggest that this substance plays a definite rôle in the fertilization reaction.

SUMMARY

1. Sperm extracts of *Arbacia*, prepared by heating and filtering sperm suspensions in sea water, irreversibly agglutinate eggs of the same species. The reaction is characterized grossly by a dense coagulation of the ova and microscopically by the appearance of an "agglutination membrane" at the periphery of the egg jelly. Sperm extracts agglutinate both jelly and cortex of unfertilized, fertilized, living or dead ova.

2. The "egg-agglutinating substance" is fixed by the jelly and removed from solution. It is found only in extracts of sperm and cannot be extracted from any other cells or tissues of the sea-urchin, is not secreted by sperm into sea water, and is not present in extracts of old sperm. The egg-agglutinating substance is colorless, non-dialysable,

colloidal, highly thermostable, disintegrates on standing, is preserved best at low temperatures, and does not give the usual protein tests. On extracting sperm with hot or cold alcohol-ether, the egg-agglutinating substance is found in the protein residue but not in the lipid extract. Mg and Ca ions are the only ions of sea water which are essential to the extraction of the egg-agglutinating substance, no specific anions taking part in the process. Sperm extracts made in distilled or tap water are inert.

3. Sperm extracts inactivate the sperm-agglutinating power of *Arbacia* fertilizin, the capacity for inactivation varying directly with the concentration of extract. The "fertilizin inactivator" has many properties in common with the egg-agglutinating substance. Loss of fertilizing power from aging sperm suspensions parallels the loss of the egg-agglutinating substance and fertilizin inactivator from extracts of these suspensions.

4. Sperm extracts block fertilization in *Arbacia* by a direct effect on each gamete:

(a) Sperm suspensions in sperm extracts rapidly lose their fertilizing power as measured by their ability to fertilize fresh eggs in sea water. The extracts thus block fertilization by a direct action on the spermatozoön.

(b) The fertilizability of jellyless eggs is greatly decreased after exposure to sperm extracts, which exert an inhibitory effect on the cortex.

(5) *Arbacia* embryos are instantly paralysed and agglutinated by sperm extracts. Eggs which are fertilized in extracts cease developing before the motile blastula stage but this effect is partially removed by transferring the eggs to sea water.

6. All attempts to activate *Arbacia* eggs by immersion in sperm extracts have been unsuccessful.

7. It is suggested that the agglutination of eggs and the inactivation of fertilizin by sperm extracts represent two effects of a single active substance extracted from sperm. Theoretical and experimental evidence is presented that this substance probably plays a definite rôle in the fertilization reaction.

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LITERATURE CITED

- COHN, E. J., 1918. Studies in the physiology of spermatozoa. *Biol. Bull.*, **34**: 167-218.

- GLASER, O., 1914. On auto-parthenogenesis in *Arbacia* and *Asterias*. *Biol. Bull.*, **26**: 387-409.
- GOLDFORB, A. J., 1929. Variation of normal germ cells. Studies in agglutination. *Biol. Bull.*, **57**: 333-349.
- HAAS, A. R., 1916. The effect of the addition of alkali to sea water upon the hydrogen ion concentration. *Jour. Biol. Chem.*, **26**: 515-517.
- HEILBRUNN, L. V., 1923. The colloid chemistry of protoplasm. I. General considerations. II. The electrical charges of protoplasm. *Am. Jour. Physiol.*, **64**: 481-498.
- IRVING, L., 1926. The precipitation of calcium and magnesium from sea water. *Jour. Mar. Biol. Ass'n, N.S.*, **14**: 441-446.
- JUST, E. E., 1922. The effect of sperm boiled in oxalated sea-water in initiating development. *Science, N.S.*, **56**: 202-204.
- JUST, E. E., 1928. Methods for experimental embryology with special reference to marine invertebrates. *Collecting Net*, **3** (No. 2): 7.
- JUST, E. E., 1930. The present status of the fertilizing theory of fertilization. *Protoplasma*, **10**: 300-342.
- KAPP, E. M., 1928. The precipitation of calcium and magnesium from sea water by sodium hydroxide. *Biol. Bull.*, **55**: 453-458.
- KOLLE, W., AND H. HETSCH, 1935. Experimental Bacteriology in its Applications to the Diagnosis, Epidemiology, and Immunology of Infectious Diseases. Seventh German Edition. Revised by J. Eyre. Vol. 1, 592 pp. Macmillan, New York.
- LILLIE, F. R., 1913. Studies of fertilization. V. The behavior of the spermatozoa of *Nereis* and *Arbacia* with special reference to egg-extractives. *Jour. Exper. Zool.*, **14**: 515-574.
- LILLIE, F. R., 1914. Studies of fertilization. VI. The mechanism of fertilization in *Arbacia*. *Jour. Exper. Zool.*, **16**: 523-590.
- LILLIE, F. R., 1915a. Sperm agglutination and fertilization. *Biol. Bull.*, **28**: 18-33.
- LILLIE, F. R., 1915b. Studies of fertilization. VII. Analysis of variations in the fertilizing power of sperm suspensions of *Arbacia*. *Biol. Bull.*, **28**: 229-251.
- LILLIE, F. R., 1919. Problems of Fertilization. The University of Chicago Press, Chicago.
- LOEB, JACQUES, 1913. Artificial Parthenogenesis and Fertilization. The University of Chicago, Chicago.
- MATHEWS, A., 1897. Zur chemie der spermatozoen. *Zeitschr. f. physiol. Chemie*, **23**: 399-411.
- PETERS, J. P., AND D. D. VAN SLYKE, 1932. Quantitative Clinical Chemistry. Vol. II. Methods. Williams and Wilkins Co., Baltimore.
- PIÉRI, J.-B., 1899. Un nouveau ferment soluble: L'ovulase. *Arch. de Zool. Expér. et Gén.*, **7**: (Notes et revues) xxix-xxx.
- ROBERTSON, T. B., 1912. On the isolation of oöcytase, the fertilizing and cytolyzing substance in mammalian blood-sera. *Jour. Biol. Chem.*, **11**: 339-346.
- SAMPSON, MARY M., 1926. Sperm filtrates and dialyzates. Their action on ova of the same species. *Biol. Bull.*, **50**: 301-338.
- WELLS, H. G., 1929. The Chemical Aspects of Immunity. Second Edition. Am. Chem. Soc. Monograph Series, No. 21. Chemical Catalogue Co., New York.
- WOODWARD, ALVALYN E., 1915. Note on the nature and source of "purple X." *Biol. Bull.*, **29**: 135-137.

EFFECT OF CERTAIN BACTERIA ON THE OCCURRENCE OF ENDOMIXIS IN *PARAMECIUM AURELIA*¹

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It was at first believed that endomixis occurs in *Paramecium aurelia* at rather regular intervals, but detailed observations have shown that there is much variation in the length of the interendomictic period. Different stocks show differences in the average interval between endomixes: thus Sonneborn (1937) showed that, under identical conditions, in the stock *R* the mean interval between endomixes was 18.3 days (66.3 fissions), while in Woodruff's long-lived stock (*W*) the mean interval was 46.3 days (110.4 fissions). Environmental conditions have been shown to alter the interendomictic period. Woodruff (1917) showed that sudden alterations in the cultural conditions bring on endomixis earlier than it would otherwise occur. Jollos (1916), by various changes in temperature and bacterial content of the culture medium, caused great changes in the length of the interendomictic period, from a minimum of 3 days to a period sufficiently great for 168 fissions to occur. Young (1917) increased the frequency of endomixis in *Paramecium aurelia* by alterations in the age of the culture medium and by changes in temperature. In *Paramecium caudatum*, Chejfec (1930) showed that the percentage of individuals undergoing endomixis in mass cultures was greater in a medium containing the bacillus coli than in hay infusion.

In the following study of *Paramecium aurelia* the frequency of endomixis and length of the interendomictic period were compared in culture media containing diverse bacteria, in different concentrations. Special attention was directed to the question whether it is primarily the unfavorable nature of conditions that brings on endomixis or increases its frequency.

A branch of Woodruff's long-lived stock of *Paramecium aurelia* was employed in the experiments. All lines of descent were derived from a single individual which underwent endomixis at room temperature during the first five-day period of the experiment (see Fig. 1). Thereafter all cultures were kept in a constant temperature box at about 25° to 26° C., except during the daily transfers.

¹ I wish to thank Dr. H. S. Jennings and Dr. T. M. Sonneborn for their help and criticism.

Five types of bacteria were employed in the different culture media, namely *Flavobacterium brunneum*, *Bacillus niger*, *B. cereus*, *B. prodigiosus*, and *B. coli*.

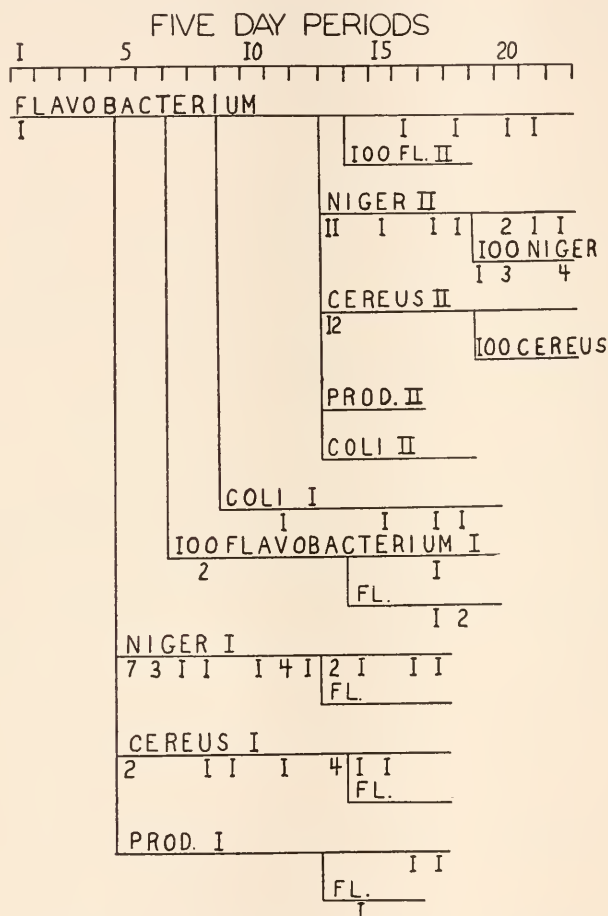


FIG. 1. Diagram of the course of the experiments. Each interval from left to right represents a five-day period. The horizontal lines show the number of five-day periods through which each culture (of 12 lines each) continued. The small numbers below the horizontal lines in certain periods give the number of lines (out of the 12) that underwent endomixis in that period. The heavy vertical lines show the source of the cultures; thus the culture "Niger I" was derived from the *Flavobacterium* culture at the end of its fourth five-day period: it continued through the seventeenth five-day period.

Flavobacterium, *Flavobacterium brunneum*; *Niger*, *Bacillus niger*; *Cereus*, *B. cereus*; *Prod.*, *B. prodigiosus*; *Coli*, *B. coli*.

Designations preceded by 100 indicate cultures in which the concentration of bacteria was 100 times as great as normal; designations not preceded by 100 indicate the normal concentration. I and II indicate Groups I and II, mentioned in the text.

The standard culture medium was that commonly employed in this laboratory. It consists of infusion of desiccated lettuce leaves, into which is introduced the green alga *Stichococcus bacillaris*, in the proportion of three 2 mm. loops of the alga (from 20 to 22-day-old slants) to 20 cc. of the filtered fluid. To this is added a 1 mm. loop of *Flavobacterium brunneum* from a one-day-old agar slant. (For details see Sonneborn, 1936.) Since *Paramecium aurelia* flourishes well in this medium, it was employed as a norm or standard for comparing the results with the other bacteria.

For the other bacteria the medium was prepared in the same manner, with the substitution of equal amounts of the other bacteria for *Flavobacterium brunneum*. The medium is buffered to prevent the production of different pH by the diverse bacteria. In some of the experiments a nutritive medium was employed in which the quantity of bacteria was 100 times as great per unit volume as in the normal or standard medium. The purpose was to determine the results of preponderance of bacteria of a certain type, so that it was not considered essential to carry on the experiments under purely aseptic conditions; this would, for experiments of this nature, be practically impossible. Tests were made at intervals to determine whether there was marked contamination. No cross-contamination of the different bacterial types employed was observed in any case. There were three types of common laboratory contaminants; when these became at all abundant in the cultures (up to 30 per cent) such cultures were rejected.

For determining how far the effects of certain bacteria in certain concentrations are harmful or unfavorable, the following criteria were used. An unfavorable effect may be shown by (1) a slowing of the fission rate, (2) an increase in the proportion of deaths occurring, (3) incomplete division, resulting in the production of partially united chains of individuals. Accordingly, observations on these points were recorded for each different type of culture (see Table I). The occurrence of endomixis in the different media was detected by the daily staining of samples from each line.

The general plan of the experiments is shown in Fig. 1, on which is likewise indicated the number of lines in which endomixis occurred in particular five-day periods. In Table I are summarized the general results of culture in the different media. The following account of results is to be read in connection with the figure and table.

From a single individual that had just undergone endomixis, twelve lines were developed as quickly as possible, by cultivation in the normal *Flavobacterium* medium described above. This culture of 12 lines in *Flavobacterium* was maintained throughout the 22 five-day periods of

TABLE I

A summary of the effects of different bacterial media upon endomixis, the number of deaths and of chain-formation in Paramecium aurelia

Group	Total number of line-days	Mean fissions per line per five-day period	Total number of deaths	Number of deaths per 100 line-days	Total number of chains formed	Number of chains per 100 line-days	Total number of lines in endomixis	Number of lines in endomixis per 100 line-days
<i>Flavobacterium brunneum</i>								
Normal Concentration	1232	10.38	10	.81	2	.16	5	.41
100 Times Normal (Group I)	748	5.27	31	4.14	26	3.48	3	.40
Group I Returned to Normal	360	9.13	3	.83	3	.83	3	.83
100 Times Normal (Group II)	236	6.09	9	3.81	9	3.81	0	0
<i>Bacillus niger</i>								
Normal Concentration (Group I)	746	8.23	6	.80	0	0	23	3.08
Group I Returned to <i>Flavobacterium</i>	291	11.92	0	0	0	0	0	0
Normal Concentration (Group II)	590	8.91	9	1.53	0	0	18	3.05
100 Times Normal	240	9.82	3	1.25	0	0	8	3.33
<i>Bacillus cereus</i>								
Normal Concentration (Group I)	750	8.69	4	.53	0	0	11	1.47
Group I Returned to <i>Flavobacterium</i>	220	8.88	0	0	0	0	0	0
Normal Concentration (Group II)	297	8.93	3	1.01	0	0	12	4.04
100 Times Normal	225	4.61	7	3.11	7	3.11	0	0
<i>Bacillus prodigiosus</i>								
Normal Concentration (Group I)	772	8.88	7	.91	7	.91	2	.26
Group I Returned to <i>Flavobacterium</i>	207	8.48	0	0	2	.97	0	0
Normal Concentration (Group II)	197	9.42	4	2.03	4	2.03	1	.51
<i>Bacillus coli</i>								
Normal Concentration (Group I)	658	9.43	15	2.28	15	2.28	4	.61
Normal Concentration (Group II)	359	8.92	8	2.23	10	2.79	0	0

the experiments; it is represented by the upper horizontal line in Fig. 1. Cultures employed in the experiments with other media were branched off from this normal culture in particular five-day periods, as shown in Fig. 1. In each of the different media a group of 12 lines was kept in progress during a number of five-day periods, as indicated in the figure. The number of "line-days" given in column 2 of Table I is the number of lines of descent (usually 12, but with occasional irregularities), multiplied by the number of days during which the lines were in progress. In some cases two different groups of 12 were tested at different times in a particular medium; these are designated as I and II in the figure and table.

The main features of the diverse experimental cultures are as follows.

FLAVOBACTERIUM BRUNNEUM: NORMAL OR STANDARD CONCENTRATION

The 12 lines in this medium were kept in progress for 22 five-day periods, which covered the entire time of the experimental cultures. Total number of line-days, 1232. Mean fission rate, 10.38 per line per five-day period. Endomixis had occurred at the beginning of the culture; it occurred again in but four of the lines, in periods 16, 18, 20 and 21 (Fig. 1)—after intervals respectively of 75, 87, 97 and 103 days (168, 180, 212 and 224 generations). The remaining 8 lines did not undergo endomixis during the rest of the 110 days of the culture. The interendomictic periods are thus very long (as was before known to be the case in this stock). Thus, as the table shows, the total number of lines found to be in endomixis during this part of the experiment was but 5 (including that in the first generation), so that the number of endomictic lines per 100 line-days was but 0.41 (Table I).

In this medium the total number of deaths was but 10, or in the proportion of 0.81 per 100 line-days. Only 2 cases of united chains (incomplete division) occurred, thus but 0.16 per 100 line-days.

High concentrations of *Flavobacterium*.—At the beginning of the seventh 5-day period a group of 12 lines (Group I) was branched off and cultivated in the same medium, but with the concentration of bacteria 100 times as great. They were continued here till the end of the 19th period. As Table I shows, this decreased the fission rate to about one-half its former rate (5.27 in place of 10.38 per five-day period): multiplied the death rate by five and the proportion of incomplete fissions (chains) by about 22. Furthermore, the size of the individuals was notably decreased, and the contained crystals became larger and more numerous. Thus this high concentration must be considered distinctly unfavorable as compared with the normal. Seven days after the trans-

fer (in period 8, Fig. 1) two of the 12 lines underwent endomixis. A third line was in endomixis in the seventeenth five-day period: almost simultaneously (in periods 16 and 18) two lines were in endomixis in the normal concentration, so that no special significance can be attributed to this.

Thus the change to the unfavorable high concentration apparently induced endomixis in 2 of the 12 lines.

At the fourteenth period a new group of 12 lines was branched off from the normal concentration to the concentration 100 times as great (100 FI II, Fig. 1): it was continued through 4 periods. The unfavorable effects were shown about as before (Table I), but endomixis was not induced.

Restoration to normal concentration after culture at the high concentration:—In the case of Group I cultivated at the hundred-fold bacterial concentration, after seven five-day periods (at the 14th period of the experiment as a whole), a group of 12 lines was restored to the more favorable normal concentration. Fission rate was restored to normal, the proportion of deaths and of chains was greatly decreased (Table I). The normal size of the animals was restored after five days. Endomixis occurred in 3 of the lines in periods 17 and 18, but this cannot be attributed to the change to the normal concentration, since in the 100-fold concentration, as well as in the original culture in normal conditions, lines were in endomixis at practically the same times (in periods 16, 17, 18, Fig. 1).

BACILLUS NIGER

At the beginning of period 5, and again at the beginning of period 13, derivatives of the normal control group in *Flavobacterium* were washed and transferred to a medium containing *Bacillus niger* in place of *Flavobacterium* (see Fig. 1, "Niger I" and "Niger II"). The niger medium roughly corresponded in concentration to that of *Flavobacterium* (one 1-mm. loop to 20 cc. of culture fluid). In both cases there was a sharp lowering of the fission rate in the first period after transfer, but this was soon partially recovered, the average fission rate being 8.23 (Group I) and 8.91 (Group II) as compared with 10.38 in the entire *Flavobacterium* culture medium, so that these differences are hardly significant. As Table I shows, none of the other criteria indicate that *B. niger* is less favorable than *Flavobacterium*. Yet endomixis was at once induced in *B. niger*—in 7 lines in Group I, in 11 lines in Group II (Fig. 1). In all there were 23 lines in endomixis in Group I of *B. niger*, 18 in Group II—as compared with but 5 in the entire course of the *Flavobacterium* culture. The number of endomictic lines per 100

line-days was in *B. niger* a little over 3, as compared with 0.4 in *Flavobacterium* (Table I). In Group I, 9 lines underwent a second endomixis while in *B. niger*, and the interendomictic interval was short, ranging from 24 days (44 generations) to 48 days (79 generations), as compared with 75 to 103 days in *Flavobacterium*. Similar relations are found in Group II of *B. niger*.

It appears clear, therefore, that culture in *B. niger* greatly increases the frequency of endomixis, and that this is not a result of unfavorable conditions, for *B. niger* appears otherwise as favorable as *Flavobacterium*.

When derivatives from Group I in *B. niger* were restored to *Flavobacterium*, after 40 days in *B. niger*, there was after a few days a rise in fission rate. No endomixis occurred in those thus restored to the normal medium.

High Concentrations of B. niger

When individuals of Group II in *B. niger* were transferred to a concentration of *B. niger* 100 times as great as normal, there was no appreciable change in fission rate, mortality, or in number of imperfect divisions. Endomixis, however, was increased, 8 lines undergoing endomixis in the 4 periods of this culture (Fig. 1). This agrees with the fact previously noted, that the presence of *B. niger* in the culture medium increases the frequency of endomixis, and that this increase is not the result of the unfavorableness of *B. niger*.

BACILLUS CEREUS

Two groups of 12 lines were transferred from *Flavobacterium* to a normal concentration of *B. cereus*, at periods 5 and 13 of the experiment (Fig. 1). Fission rate was very slightly decreased, and the animals were on the average a little smaller in size than in *Flavobacterium*, but mortality did not increase and there were no imperfect fissions, forming chains. Thus this bacillus can hardly be considered appreciably harmful. Yet, as in *B. niger*, endomixis was definitely increased in frequency. There were eleven endomixes in the 12 lines of Group I, 12 in those of Group II, while in the same periods the lines in *Flavobacterium* underwent but 4 endomixes. Seemingly *B. cereus* is less effective than *B. niger* in producing endomixis, since none of the lines in *B. cereus* underwent a second endomixis, while a number of those in *B. niger* did so, though the number of periods was the same for the two types of culture.

Restoration of lines of *B. cereus* culture to *Flavobacterium* did not result in the production of endomixis.

High Concentration of B. cereus

Derivatives of Group II in normal *B. cereus* were transferred at the nineteenth period to a concentration of *B. cereus* about 100 times as great (Fig. 1). This decreased the fission rate by about one-half, increased the mortality, and the formation of abnormal chains, and caused the production of large crystals in the cytoplasm. Conditions were clearly unfavorable, but endomixis was not induced.

BACILLUS PRODIGIOSUS

At periods 5 and 13, two groups (I and II) were derived from the normal control cultures in *Flavobacterium*, and were cultivated in the normal concentration of *B. prodigiosus*. Fission was slightly lowered and the number of abnormal chains slightly increased (Table I); also a few individuals without macronuclei were observed. Thus the medium containing this organism must be considered slightly unfavorable. No increase in the frequency of endomixis was produced.

BACILLUS COLI

Groups I and II, derived in the usual way from the *Flavobacterium* cultures at the beginning of periods 9 and 13, showed little change in fission rate, but there was a considerable increase in the proportion of deaths, and of abnormal divisions (chains). Also numbers of individuals without macronuclei occurred. In Group I, four cases of typical endomixis occurred (Fig. 1). In addition, there occurred in most of the lines irregular fragmentation of the macronucleus. This began in both groups a few days after the transfer to *B. coli*, and continued for about 10 days. The fragments varied from day to day in size and number. They were scattered through the cytoplasm, or sometimes were in pockets of the macronucleus. The macronucleus was frequently present in two pieces; often it exhibited signs of activity, in the possession of projections of various sizes. Some of the stages closely resembled figures presented by Diller (1936, p. 37) for the process of hemixis in his types *B* and *C*. This fragmentation was in most cases not followed by the typical climax stage of endomixis, though it was in one of the lines. In other cases, after the disappearance of the fragments, the macronucleus remained normal. Such fragmentation occurred only in media containing *Bacillus coli*.

SUMMARY

1. In the long-lived stock of Woodruff, cultivated in a standard medium containing a normal concentration of *Flavobacterium brunneum*,

endomixis occurred only at very long intervals, of 75 to 109 days, or more. Increase in the quantity of *Flavobacterium* to 100 times the normal concentration made the conditions very unfavorable, but hardly increased the incidence of endomixis.

2. Substitution of *Bacillus niger* for *Flavobacterium*, in the same proportions, brought on endomixis and increased its frequency, although it did not make the conditions unfavorable. Increasing by 100-fold the concentration of *B. niger* caused a further increase in the frequency of endomixis, but still without making the medium unfavorable.

3. *Bacillus cereus* was not appreciably unfavorable in its action on *Paramecium*, yet like *B. niger* it increased the frequency of endomixis. Its tendency to induce endomixis appears to be slightly less than that of *B. niger*. Increasing the concentration of *B. cereus* 100-fold made conditions very unfavorable, but did not increase the tendency to endomixis.

4. *Bacillus prodigiosus* is slightly unfavorable as compared with *Flavobacterium*, but it does not increase the frequency of endomixis.

5. *Bacillus coli* is unfavorable as compared with *Flavobacterium*. It produces a tendency to fragmentation of the macronucleus, usually not followed by complete endomixis.

6. Thus in these experiments there is no correlation between the unfavorable effect of certain bacteria and their tendency to produce endomixis. Certain species (*Bacillus niger* and *B. cereus*) are not unfavorable to *Paramecium aurelia* yet (in this stock at least) they much increase the frequency of endomixis. Certain other conditions that are unfavorable (particularly high concentrations of *Flavobacterium*) do not increase the frequency of endomixis.

LITERATURE CITED

- CHEJFEC, M., 1930. Zur Kenntnis der Kernreorganisationsprozesse bei *Paramecium caudatum*. *Arch. f. Protist.*, **70**: 87-118.
- DILLER, W. F., 1936. Nuclear reorganization processes in *Paramecium aurelia*, with descriptions of autogamy and "hemixis." *Jour. Morph.*, **59**: 11-67.
- JOLLOS, V., 1916. Die Fortpflanzung der Infusorien und die potentielle Unsterblichkeit der Einzelligen. *Biol. Centralbl.*, **36**: 497-514.
- SONNEBORN, T. M., 1936. Factors determining conjugation in *Paramecium aurelia*. I. The cyclical factor: the recency of nuclear reorganization. *Genetics*, **21**: 503-514.
- SONNEBORN, T. M., 1937. The extent of the interendomictic interval in *Paramecium aurelia* and some factors determining its variability. *Jour. Exper. Zööl.*, **75**: 471-502.
- WOODRUFF, L. L., 1917. The influence of general environmental conditions on the periodicity of endomixis in *Paramecium aurelia*. *Biol. Bull.*, **33**: 437-462.
- YOUNG, R. T., 1917. Experimental induction of endomixis in *Paramecium aurelia*. *Jour. Exper. Zööl.*, **24**: 35-53.

CONTRIBUTIONS TO THE STUDY OF DEVELOPMENT OF THE WING-PATTERN IN LEPIDOPTERA

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INTRODUCTION

The mechanism of pattern determination in Lepidoptera wings has long been a favorite model for the study of gene action. Every study of developmental processes is concerned with the study of development of manifold structure, which finds its best object in the study of pattern, e.g. the pattern of a butterfly-wing. Goldschmidt tried the first successful analysis of the factors responsible for the development of this pattern and expressed it in his theory of different velocities of developmental processes. Although most parts of his analysis could be experimentally checked and extended by Kühn and his school, the decisive observations ("relief-stage") of Goldschmidt's work were either not found, misinterpreted, or doubted by several investigators. An additional and more extended investigation of those stages was therefore necessary and will be furnished in this paper.¹

PREVIOUS EXPERIMENTS ON THE DEVELOPMENT OF THE WING-PATTERN

Goldschmidt's Investigations

In his book "Physiologische Theorie der Vererbung" (1927), Goldschmidt contributed an extensive analysis of the pattern problem.

A study of the development of the individual wing furnished the most decisive point in this analysis. Goldschmidt (1920, 1923) observed that the later wing-pattern is already completely represented in the young pupal wing, long before pigmentation takes place. It can be made visible by detaching unpigmented pupal wings from the body and allowing them to dry. After a short time a relief was visible on the former homogeneous-looking wing.

Later white scales became erected and formed the raised regions and later dark scales collapsed and formed the depressed regions. The whole relief represented the later wing-pattern in every detail. He observed this "relief-stage" in *Platysamia cecropia* L., *Lymantria*, *Thais*

¹ Acknowledgment for help and advice is due to Professor R. Goldschmidt and to the United States Government for assistance rendered through its Works Progress Administration Official Project No. 465-03-3-192.

and several other species. Goldschmidt assumed that in certain parts of the pattern the scales are still soft bags filled with blood, which collapse when dried out. In other districts, however, the scales are already chitinized and do not collapse when dried out. Because the later dark scales are consequently softer at this point of development than the later white scales, he concluded that later dark scales exhibit a slower development than later white scales. He arrived at the conclusion that the different velocities of development for the different parts of the wing were primarily responsible for the formation of wing-pattern.

According to his theory the different velocities of development start with the mosaic of the epidermis cells and their product, the scales which develop with different speed in different parts of the wing. A section through the young pupal wing in a certain stage would accordingly show us different parts of the wing in different stages of development.

At the same time different chemical substances will be present in the body, which if deposited in the scales will determine the color. These substances, like tyrosine, carotene etc., can be end-products of metabolic processes and a special production of them for the coloration-processes is not necessary. The deposition of these substances in the scales can be dependent on a certain colloidal stage of the chitin. If at a certain stage in the development tyrosine is present, it will only be deposited in those scales which represent a certain condition of the chitin at this moment, which means only a certain part of the pattern. Other parts of the pattern will react according to their stage of chitin-development and the pigmentation substances present. The development of a very complicated pattern can thus be explained by a very simple mechanism.

Goldschmidt (1920, 1927) then tries to trace these processes back in development and discusses the determination-points for these differences in developmental velocities ("sensitive period," determination-stream, Liesegang phenomenon).

The Investigations of Kühn and His Co-workers

Kühn and his co-workers tried to attack the same problems and furnished further experimental material. They worked with the flour-moth, which shows a pattern consisting of dark and light pattern-elements which are partly symmetrical (see Fig. 1). The various parts of the pattern do not only contain different amounts of pigment but the shape and size of the scales in various districts differ also. Therefore, the determination of the pattern is here not only a process of distributing different amounts of pigment but a morphogenetic process as well.

Feldotto (1933) determined the sensitive periods for this pattern and showed that heat-treatment at the pupal age of 12-72 hours was

able to modify the development of the different parts of the pattern. Each system of the pattern has its particular time of maximal modification at different stages of development.

Kühn and von Engelhardt (1933) cauterized the young pupal wing and thus were able to show that a determination-stream spreads over the wing from 48–60 hours of pupal age.

A period of particularly distributed cell divisions can be considered as the first reaction to the process of determination. These cell divisions were first described by Köhler (1932). He reported that from 36–84 hours of pupal age a great number of cell-divisions are visible which have their maximal distribution in districts of subsequent dark pattern elements while subsequent light pattern districts show few cell-divisions. The distribution of cell-divisions therefore resembles the subsequent pattern of pigmentation and Köhler consequently called it mitosis-pattern. A mitosis-pattern seems to be the predecessor to the later wing-pattern.

Braun (1936) examined the mitosis-pattern more closely with special regard to its rôle in the development of the wing-pattern.

He showed that this mitosis-pattern is visible from 36–148 hours of pupal age. Subsequent dark pattern-districts always represent maxima of mitotic divisions, while the minima are in later light pattern-districts during this period. The mitosis-pattern spreads in a wave-like fashion over the wing from the proximal base to the distal margin. Two such mitosis waves are distinguishable during the period of mitotic division. The first one goes over the wing from 36–84 hours, and represents cell-divisions of hypodermis-cells and the first division of the scale-building cells. The second wave starts at 84 hours and ends at 148 hours and represents the second division of the scale-building cells. When the first proximal divisions occur we find no mitosis in distal parts of the wing, with the exception of the cell-divisions of the later marginal scales, which start developing earlier and continue at a faster rate throughout the whole wing-development. Furthermore, it could be shown that this mitosis-pattern is the first sign of a completed determination of the wing-pattern.

Then an attempt was made to investigate the rôle of this mitosis-pattern in the development of the wing-pattern. It could be shown that the districts of mitosis-maxima (presumptive dark districts) represent districts of greater intensity of cell-division. Throughout the stages of development following the mitosis-period, later dark districts show a greater number of cells per unit than later white districts. The size of cells in the presumptive dark districts is accordingly smaller. This observation could be checked on the adult wing also, where one finds

more scale-building cells per unit in dark districts than in light districts. In addition it was found that all scales grow out simultaneously. From these observations it was concluded that a different intensity of cell-division was primarily responsible for the formation of the pattern. The principle of different velocities of development was discarded in favor of the principle of different intensity of cell-division as the primary factor in the development of a pattern. Köhler and Feldotto (1937) again tried to discard the existence of different developmental velocities for Lepidoptera in a recent paper. Their argument is based on their failure to find the scales of different districts in different stages of growth during experiments on scale-development. Furthermore, it had not been possible to observe the relief stage in *Ephestia*.

In response to these views expressed by the *Ephestia*-workers, Goldschmidt showed in recent publications (1938) that the observations made on *Ephestia* are in perfect harmony with his discoveries. The fact that we find more cell-divisions in certain districts during the mitosis-period means that the cells in these districts have not yet reached the end of their multiplication period. These districts are the later dark districts, they show a retarded development not only in the relief-stage but at this early stage as well. The cells in later white districts show less cell-divisions because they have reached the end of their multiplication period earlier and therefore differentiate faster than the later dark cells. (An investigation concerning the number of divisions which a cell undergoes in later dark or in later white districts would probably prove this explanation. Such an investigation is now under way.) The observation of the simultaneous growing out of scales does not interfere with this theory, because the different speed of differentiation of the scales in the different districts does not necessarily mean different growth. The process of growth might be nearly identical for all wing areas, while the different speed of development should be noticeable by differences in the histological structure of the scales, which would be hard to examine at this early stage.

To test these views it was necessary to undertake a more detailed investigation of the relief stage, where the different velocity of development for the different pattern districts was demonstrated so clearly. Furthermore, an attempt had to be made to find additional evidence for the different speed of development at stages previous to the relief stage. The relief-stage had to be found in *Ephestia*, where the mitosis-period had been found. Finally the mitosis-period had to be observed in other Lepidoptera, where the relief-stage had been demonstrated previously. The results of these investigations, which fully confirmed the validity of Goldschmidt's views, are described in the following section.

NEW INVESTIGATIONS ON THE DETERMINATION OF THE WING-PATTERN

Material and Methods

Ephestia kühniella Zeller, *Platysamia cecropia* L. and *Papilio ajax* L. were the objects of the experiments to be described. *Ephestia* cultures were kept according to the method described by Kühn and Henke. *Cecropia* and *Papilio* pupae were obtained in the fall and stored in a refrigerator, from which they were put into an incubator at any time desired. They started development as soon as they were placed in a warmer temperature. All pupae developed at 25°. Fifty pupae of *Platysamia cecropia*, fifty pupae of *Papilio* and approximately one hundred and fifty pupae of *Ephestia* were used for these investigations.

The Appearance of the Relief Stage

As pointed out before, *Ephestia*-workers have never been able to find the stage in the pupal-development which is decisive for the theory of different developmental velocities, namely the relief stage. Therefore the first step was to hunt for this decisive stage in the development of the flour-moth. In order to become acquainted with the stage in question, the author repeated Goldschmidt's (1920) experiments on the *Cecropia* moth and *Papilio*. The observations confirmed Goldschmidt's results completely.

Platysamia cecropia L.—The wings were removed from the pupae at a time when no pigmentation had yet taken place, the best time being one to two days before pigmentation. They were then placed on a slide and allowed to dry. A relief appeared which showed subsequent white parts erect, subsequent dark parts collapsed. This stage is relatively short, approximately 3–5 per cent of the whole pupal stage, and was observed on ten *Cecropia* wings.

The relief of the *Cecropia*-wing is especially clear in the distal parts where the dark and yellow half-moon is located on the pigmented wing. (For excellent photos of this stage see Goldschmidt's 1920 paper.)

Papilio ajax L.—The same stage was easily found in *Papilio ajax*. Here too the relief-stage takes 3–5 per cent of the complete time of pupal development. It is very clear all over the wing, corresponding to the mature wing-pattern, and appears approximately twenty minutes after the wing has been put on a slide for drying. The subsequent light parts of the wing are indicated by clearly erected parts, the subsequent dark pigmented districts of the wing are all collapsed. This stage could be observed in fourteen wings which were removed from the pupae one to two days before pigmentation started. The different unpigmented pattern districts of the wings were visible even without drying. If the light

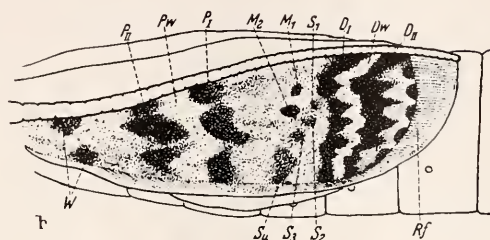
was shone in a certain way on the still attached and living wing, the districts of subsequent dark pigmentation and light pigmentation were clearly distinguishable by what appeared to be a difference of consistency of the areas in question. This difference can be demonstrated even more clearly, if one puts the wing into water. Again the districts of subsequent different pigmentation can be easily distinguished on the completely unpigmented wing. The action of two time factors for this period could be observed in *Papilio*. (1) At the moment pigmentation started, the relief disappeared in pigmented parts, while unpigmented parts showed the relief distinctly. (2) It is known that the hind wing always shows a faster development than the fore wing (e.g. pigmentation). Consequently the relief was already present on the hind wing, while all scales were still soft on the fore wing. The relief of the hind wing disappeared earlier because pigmentation set in earlier. (Pictures of the relief stage in species closely related to *Papilio* can be found in Goldschmidt's 1923 paper.)

Ephestia k.—The corresponding stage was found without much difficulty in *Ephestia* at eight to nine days of pupal age. That it had never been noticed before is surprising. The wing of the pupa was easily detached after the surrounding chitin was broken and carefully removed. Then the wing was placed on a slide and allowed to dry. After about fifteen minutes the two later dark bands appeared clearly as collapsed parts on the dried wing (Figs. 1 and 2). The action of an additional time-factor could be observed on these wings. They showed that the relief-stage proceeds like many other processes in the development of the flour-moth (mitosis-pattern, pigmentation) in a wave-like fashion from the proximal base to the distal margin of the wing. In early stages the relief of the proximal band only was visible, while in distal parts all scales were collapsed (Fig. 2 *a*). A little later the whole wing showed the relief, scales both of the proximal and distal bands being collapsed (Fig. 2 *b*). The marginal scales were an exception. They always exhibited a faster development, first noticeable during the mitosis period, and they were already erected in distal "white" parts in the early relief-stage. As in *Papilio*, the districts of subsequent different pigmentation were visible on the undried wing if the light was reflected in a certain way, as well as when the wing was put into water. These observations were based on a study of approximately sixty pupae.

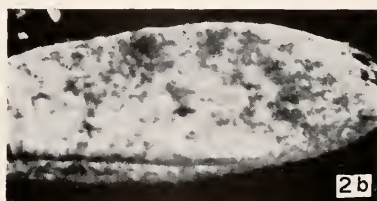
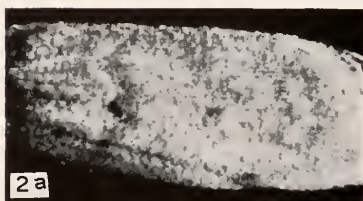
The Chitinization of Scales during Pupal-development as Proof of the Different Velocity of Development

Sections.—The fact that no regional differences in the speed of outgrowing scales could be detected has been mentioned before (Köhler,

1932; Braun, 1936; Köhler and Feldotto, 1937). It was necessary to make sections of the wings in the relief stage to see if erect scales show a morphological difference at this stage. Such sections were made from



1



3

FIG. 1. Pigmented pupal wing of *Ephestia kühniella*. P_I , P_{II} proximal dark bands, D_I , D_{II} distal dark bands, M_I , M_{II} middle-spots.

FIG. 2a-b. Relief-stage of the pupal wing of *Ephestia kühniella*. a, early relief-stage showing the proximal band clearly, b, later relief-stage showing proximal band, distal band and five marginal spots. $\times 9$.

FIG. 3. *Papilio ajax*. $\times \frac{2}{3}$.

eight *Cecropia* wings (through the district of the "half-moon"), from twelve *Ephestia* wings, and six *Papilio* wings, but no morphological differences in the stage of development of collapsed or erect scales of different forms were noticed. In many Lepidoptera white and dark areas are composed of scales of different forms, correspondingly later dark parts showed larger scales on the *Cecropia* wing than later white parts.

Consequently the different speed of differentiation of the scales in the different pattern districts does not manifest itself by different stages of growth. Growth seems to be the same for all parts of the wing. The difference therefore has to be sought in different velocities of the chitinization process of the later white and dark scales at this stage as suggested by the soft condition of later dark areas.

Chemical Tests.—In fact, such a difference could be detected by chemical reactions. There is not much known about the chemistry of chitin, but P. Schulze (1922) described a differential reaction for hard and soft chitin. The object is first saturated with iodine for a short time, blotted with filter paper, and finally covered with a solution of ZnCl_2 . The reaction will show very soon but disappears after some time. Hard chitin will show a violet-brown color, soft chitin, a light brown. Ten unpigmented *Cecropia* wings were treated accordingly and showed a distinct reaction. Subsequent white parts appeared dark; subsequent dark parts appeared light. This experiment exhibits clearly that the different parts are found in different stages of chitinization. The same experiment was repeated with six pupae of *Papilio* and twenty pupae of *Ephestia* and always resulted in a clear pattern negative. Even the sections which showed no morphological difference reacted distinctly to this chitin test. The chitin reaction worked during the whole relief stage. It would have been desirable to check this test by making the same experiment with an adult pigmented wing. In this case all scales should show the violet-brown reaction. However, it was not possible to extract the pigment from an adult wing, which would have been necessary in order to obtain a bleached wing on which the reaction would be visible. Several other chitin reactions were tested, but none of them gave as clear a result as did the iodine-zinc reaction.

Test with Polarized Light.—After it had been proven that the different speed of development actually can be shown to be the different velocity of chitin hardening, an experiment to test the refraction of the differently chitinized scales was tried. Adult scales are doubly refracting, and it was hoped that one could find a stage where one kind of scales was singly refracting. Scales from different regions were investigated under crossed Nicols, but no clear results were obtained. It was men-

tioned before that the scales from different regions have different forms, and this difference may add to different refraction effects.

Tyrosine Reactions.—The next experiment was an attempt to produce premature pigmentation artificially in order to watch the reaction of the different regions. Studies on the chemistry of melanin formation by several authors have shown that two components are necessary for pigmentation: a chromogen and an oxydase. Oxydase (e.g. tyrosinase) is always present in the blood of Lepidoptera as may be easily shown, while the chromogen is supposed to enter the wing at a definite stage of development (the time of pigmentation). Oxydase and oxygen together produce a melanin pigment. If the blood of Lepidoptera contains tyrosinase it should be possible to produce a dark pigment on the yet unpigmented wing by providing the necessary chromogen, e.g. by soaking the unpigmented wing in a solution of tyrosine.

(1) *Papilio ajax*.—An unpigmented pupal wing of *Papilio* at the time of the relief stage was put in a saturated solution of tyrosine. At the stage in question the pupal wing is always folded in a definite manner, which allows for the subsequent expansion of the wing after hatching. The wing is compressed in length and width to one-third of its actual size. This results in a wave-like appearance of the epithelium, with the scale-bearing epithelium as the crest and the epithelium without scales as the trough. (These folds do not interfere with the observation of the relief-stage on the pupal wing.) After immersion in the solution for four hours at 30°, the wing exhibited the following characteristics: it was completely stretched like the wing of the imago and showed the complete pattern of an adult pigmented wing; subsequent dark parts were dark, subsequent light parts were white (unpigmented) (Figs. 3 and 4). Microscopical examination of wings which had been treated with tyrosine revealed that the pigment is actually deposited in the scales. It is, however, not as black as the normally deposited pigment in the wing of the hatched butterfly. This experiment shows that a certain viscosity of the chitin is necessary for the deposition of a pigment in colloidal solution. After a scale has reached a certain point of hardening it cannot deposit any more pigment. The tyrosine can react with the tyrosinase in the still soft scales of subsequent dark parts, but no reaction can take place in the hardened scales of subsequent light parts. The tyrosine experiment demonstrates clearly how the different speed of hardening in different regions leads to the pattern of pigmentation.

Pupal wings of different age were treated accordingly to investigate the length of the stage during which a tyrosine reaction is possible. Very young and soft pupal wings, those on which the scales have just started to grow, show after treatment with a tyrosine solution a light

gray color all over the wing with very faded contrasts of dark and light areas. The veins, however, and the marginal scales show a dark black pigment (Fig. 5). A control wing, dried at the same stage, shows all scales collapsed. In a later stage a dried-out wing will still show all scales collapsed, but if it is treated with tyrosine, a pale pattern will become visible, and subsequent dark parts will show black reaction, while subsequent light parts will not show any reaction with tyrosine (Fig. 6). With the help of the tyrosine reaction we thus can trace back the differential velocity of chitinization to a very early point in the scale de-

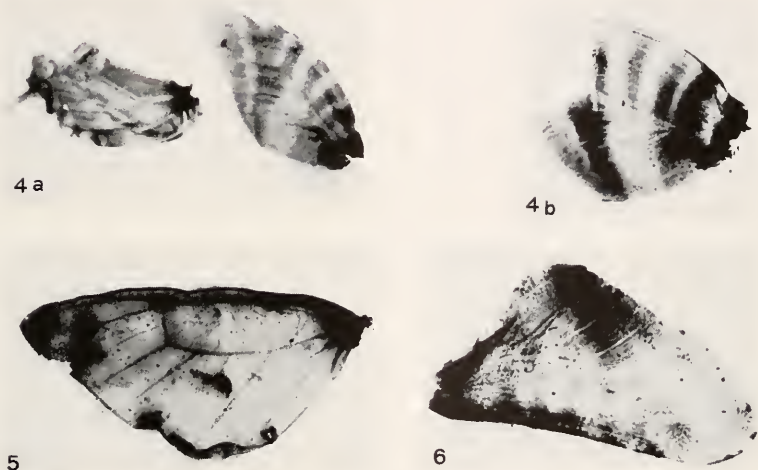


FIG. 4a-b. Pupal wings of *Papilio ajax* after treatment with tyrosine ca. 1 day before pigmentation begins to set in. a, fore wing and hind wing $\times 1\frac{1}{2}$, b, part of the fore wing $\times 2\frac{1}{2}$.

FIG. 5. Pupal wing of *Papilio ajax* after treatment with tyrosine ca. 2 days before pigmentation begins to set in. $\times 2\frac{1}{2}$.

FIG. 6. Pupal wing of *Papilio ajax* after treatment with tyrosine ca. 3 days before pigmentation begins to set in. $\times 2\frac{1}{2}$.

velopment. Long before the relief stage we are able to produce the later pattern, which means that before we can see the roughly morphological difference of the chitinization in the relief stage, we are able to demonstrate it by chemical means. The chitin of subsequent white scales either hardens earlier and does not allow the tyrosine to penetrate, or at these moments the white scales do not contain sufficient blood which, as carrier of the oxydase, is necessary for the reaction with the tyrosine. The first explanation seems more plausible. All the wings which showed the tyrosine reaction still have to be examined microscopically more carefully to complete our knowledge of the details of this process. But

the fact remains that from a very early stage, the different velocities of the different districts can be shown long before we can observe collapsed or erected scales after exposure of the wing to air. The tyrosine reaction can be performed until normal pigmentation sets in on the wing and always gives a clear positive of the pattern. Eighteen wings of *Papilio* were used for these observations and *Papilio ajar* has proved to be an excellent object for these studies.

(2) *Ephestia k.*—The tyrosine reaction was tested on thirty flour-moth wings but did not show the bands distinctly, probably because the parts of the pattern of the *Ephestia* wing are not clear and limited enough.

It has been mentioned above that the pupal wings which are folded when removed from the pupae will always unfold after they have been placed in tyrosine. No other solution tested produced this phenomenon. Pupal wings from several species, among others pupal wings of *Drosophila*, were put in a tyrosine solution, and they always unfolded after one or two hours. This fact might be of great help for other embryological work in insects.

Dissolution of the Scales.—The next test was based on the following consideration. The scales show a distinctly different chitinization at the relief stage. It is easy to remove scales from the collapsed or erect parts. If the scales from both parts could be dissolved, the harder scales should need a longer time for dissolving. Mature scales on the wing of a hatched butterfly should show the same degree of chitinization in all parts, and white and dark scales should need the same time for dissolution. Such an experiment could furnish another proof that the different chitinization during development is really an expression of the differential speed of some developmental processes. All our experiments have a greater meaning if it can be proved that all scales are chitinized equally on the adult wing.

The experiments proved that this idea was right.

Papilio ajar scales from subsequent light and dark parts were dissolved in concentrated sulfuric acid. Subsequent dark scales start to dissolve as soon as touched by the acid. Subsequent white scales first show air bubbles when put into H_2SO_4 , and then start to dissolve slowly (ca. 12 minutes). If the same experiment is repeated with scales from a mature pigmented wing, the white scales will show the same reaction as they did during the relief stage. This means they were already mature at this age. Dark pigmented scales, however, now show a longer resistance to the acid and start to dissolve only after hours of treatment. The prolonged time of the dark scales as compared with that of the white scales is probably due to the pigment deposition in the dark scales.

Scales from the same wing which were utilized for the observation of the relief-stage were used for this experiment. The same results were obtained in fifteen successive tests.

The Mitosis-pattern in Cecropia and Papilio ajax

In order to construct a general picture of the elements important for the pattern formation it was finally necessary to show that all the stages discussed (mitosis-pattern, different chitinization, relief-stage) are of general occurrence. Only the general presence of all stages, in particular relief-stage and mitosis-period, allows us to correlate them. The mitosis-pattern of *Ephestia* has been clearly demonstrated and closely investigated previously (Braun, 1936). The same kind of mitosis-pattern has been found now in ten wings of young *Cecropia* pupae, and follows the same principles as in *Ephestia*.

(The youngest *Papilio* pupae which we had in our laboratory were already too old to show the period of cell division.)

These observations confirmed that the mitosis-pattern, as well as the relief stage, is a general stage in the development of the wings of the Lepidoptera.

DISCUSSION

The new evidence gained by these experiments confirms Goldschmidt's views completely. The tyrosine reaction, the relief stage and its chitin reactions, and finally the test of dissolving scales, leave no doubt that we are dealing with different velocities of development for the different parts of the pattern. This difference turned out to be a difference in the hardening or maturing of chitin. Under these circumstances it is only logical to interpret the mitosis-pattern in these terms too. We have seen that all the stages of development for different parts of the pattern are generally present in such different species of Lepidoptera as *Ephestia*, *Papilio ajax* and the *Cecropia* moth. *Ephestia*-workers neglected Goldschmidt's views on the basis of not finding different districts of the pattern in different stages of development, but they only considered growth, which does not seem to be an exact indicator for the different velocities. The indicator for these velocities seems to be of a purely histological nature. We have been able to demonstrate such an indicator in the process of chitinization of the scales. In interpreting the mitosis-pattern in the same terms as the subsequent stages of development we are aided by the similarity of time factors in both stages. The mitosis-period and the relief stage proceed wave-like proximally to distally over the wing. In both periods we find the mar-

ginal scales advanced in development as compared with all the other scales.

Based on former investigations and our new experiments we can describe the development of the pattern in Lepidoptera as follows:

In the very young pupal wing, districts of different visible as well as physico-chemical structure are present, partly produced by the localization of tracheas, veins and different surface conditions, perhaps also by processes in which a Liesegang phenomenon is involved. At a certain time in development a determination stream (or more than one) spreads over the wing and is distributed pattern-like according to the chemico-physical conditions present in the substrate and dependent upon the points of origin and directions of the stream or streams. In the case of *Ephesia* it can be shown that the subsequent dark districts are determined in the areas where the determination-stream stops. At any time before the occurrence of the determination-stream the subsequent pattern can be altered by extreme environmental conditions like heat treatment. Different parts of the pattern can be altered at definite limited periods, the "sensitive periods." Different phenotypes or genotypes are produced by the change of time-action of the determination-stream. The determination-stream determines districts with different speeds of differentiation. These different velocities of development are first visible in the mitosis-period which follows the period of the determination-stream. During this mitosis-period more cell-divisions are visible in later dark districts than in subsequent white districts, thus forming a mitosis-pattern, equal to the later pattern of pigmentation. The cells of the subsequent dark parts, which show a more intensive division process, have not yet reached the end of their multiplication period, in contrast to the subsequent white parts which enter their period of differentiation first. The mitosis-pattern spreads over the wing in the form of a mitosis-wave from the proximal base to the distal margin of the wing. Two such mitosis-waves can be observed. The first one is composed of cell-divisions of the hypodermis-cells and the first divisions of the scale-building cells. The second one is composed of the second cell-divisions of the scale-building cells. The different velocity of development for the different parts of the pattern can be observed again some time before pigmentation sets in. It can be made visible by the tyrosine reaction, the relief stage and its chitin reactions, and by the test of dissolving scales. These tests show that the subsequent dark parts, which have already been retarded during the mitosis-period, develop slower up to the time of pigmentation. The velocity of hardening of the chitin is different for the different parts of the pattern, and this difference finally leads to the pattern of pigmentation. Since a pigment is de-

posited in the chitin in colloidal solution, it can only be deposited as long as a scale has not reached a certain point of hardening. At a certain time of development pigment is present in the body and the subsequent dark parts, being still soft at this time, will deposit pigment; the subsequent white scales on account of their faster development, which has led to a harder chitinization, cannot deposit any pigment at this point. In this way the different velocities of differentiation of different areas of the wing throughout the pupal development starting from the time of the determination stream will lead to the pattern of pigmentation.

SUMMARY

1. Goldschmidt's observations and ideas on the problem of different velocities of development for different parts of a pattern are described. The experiments of Kühn and his school and their negative views in regard to the idea of different velocities of developmental processes are summarized.

2. New observations confirm the existence of the "relief-stage" in the pupal wing of *Platysamia cecropia* and *Papilio ajax*, as well as in *Ephesia k.*, where it has not been previously observed.

3. It was observed that the relief-stage proceeds over the pupal wing from the proximal base to the distal margin in a wave-like fashion as is the case with the mitosis-pattern. The relief stage and the mitosis-pattern appear on the hind wing earlier than on the front wing.

4. No difference in growth of the scales was observed in sections through the wing during the relief-stage.

5. With the help of a chitin-reaction it could be shown that during the relief-stage the subsequent light parts of the wing were more chitinized than the subsequent dark parts.

6. A test with polarized light during the relief-stage gave no clear results.

7. With the help of artificial pigmentation (tyrosine-reaction) a complete pattern can be produced on the still unpigmented wing long before the relief-stage could be observed.

8. This tyrosine-reaction shows that a certain condition of the chitin is necessary to deposit pigment in the scales. Subsequent light parts of the wing are more chitinized at the time of pigmentation than subsequent dark parts and therefore cannot deposit any pigment. This experiment proves that the different velocities of development of the different parts of the pattern lead to the pattern of pigmentation.

9. By dissolving scales from subsequent dark and light districts in H_2SO_4 during the relief-stage and dark and light scales on the mature

wing, it could be actually shown that the scales are differently chitinized at the time of pigmentation and equally chitinized on the mature wing.

10. The similarity of processes during the mitosis-period and the relief stage and their general appearance in different species of Lepidoptera allow us to correlate them. Both stages express the different velocities of developmental processes for different parts of the wing, which finally produce the pattern of pigmentation.

LITERATURE CITED

- BRAUN, WERNER, 1936. Über das Zellteilungsmuster im Puppenflügelepithel der Mehlmotte *Ephestia kühniella* Z. in seiner Beziehung zur Ausbildung des Zeichnungsmusters. *Arch. f. Entw.-mech.*, **135**: 494.
- FELDOTTO, W., 1933. Sensible Perioden des Flügel-musters bei *Ephestia kühniella* Zeller. *Arch. f. Entw.-mech.*, **128**: 299.
- GOLDSCHMIDT, R., 1920. Untersuchungen zur Entwicklungsphysiologie des Flügel-musters der Schmetterlinge. *Arch. f. Entw.-mech.*, **47**: 1.
- GOLDSCHMIDT, R., 1923. Einige Materialien zur Theorie der abgestimmten Reaktionsgeschwindigkeiten. *Arch. mikr. Anat.*, **98**: 292.
- GOLDSCHMIDT, R., 1927. Physiologische Theorie der Vererbung. Berlin.
- GOLDSCHMIDT, R., 1938. Physiological Genetics. McGraw-Hill Book Co.
- KÖHLER, W., 1932. Die Entwicklung der Flügel bei der Mehlmotte *Ephestia kühniella* Zeller, mit besonderer Berücksichtigung des Zeichnungsmusters. *Zeitschr. Morph. u. Okol. Tiere*, **24**: 582.
- KÖHLER, W., u. W. FELDOTTO, 1935. Experimentelle Untersuchungen über die Modifikabilität der Flügelzeichnung, ihrer Systeme und Elemente in den sensiblen Perioden von *Vanessa urticae* L., nebst einigen Beobachtungen an *Vanessa io* L. *Arch. Jul. Klaus-Stiftg.*, **10**: 313.
- KÖHLER, W., u. W. FELDOTTO, 1937. Morphologische und experimentelle Untersuchungen über Farbe, Form und Struktur der Schuppen von *Vanessa urticae* und ihre gegenseitigen Beziehungen. *Arch. f. Entw.-mech.*, **136**: 313.
- KÜHN, A., 1932. Zur Genetik und Entwicklungsphysiologie des Zeichnungsmusters der Schmetterlinge. *Nach. Ges. Wiss. Göttingen, Math.-physik. Kl.*, p. 312.
- KÜHN, A., u. M. VON ENGELHARDT, 1933. Über die Determination des Symmetriesystems auf dem Vorderflügel von *Ephestia kühniella* Z. *Arch. f. Entw.-mech.*, **130**: 660.
- KÜHN, A., u. K. HENKE. Genetische und entwicklungsphysiologische Untersuchungen an der Mehlmotte *Ephestia kühniella* Zeller I-VII. *Abh. Ges. Wiss. Göttingen, Math.-physik. Kl.*, **15**: H. 1 (1929); VIII-XII, **15**: H. 2 (1932); XIII u. XIV, **15**: H. 3 (1936).
- SCHULZE, P., 1922. Über Beziehungen zwischen pflanzlichen und tierischen Skelettsubstanzen und über Chitinreaktionen. *Biol. Zentralbl.*, **42**: 388.
- STOSSBERG, MARGARETE, 1937. Über die Entwicklung der Schmetterlingsschuppen. *Biol. Zentralbl.*, **57**: 393.
- SÜFFERT, FRITZ, 1937. Die Geschichte der Bildungszellen im Puppenflügelepithel bei einem Tagsschmetterling. *Biol. Zentralbl.*, **57**: 615.

THE RELATIONSHIP BETWEEN THE PITUITARY GLAND AND THE GONADS IN FUNDULUS

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INTRODUCTION

From the extensive work on mammals, birds, reptiles and amphibians it is clear that in these forms the anterior lobe of the pituitary gland is an essential factor in the control of the sex cycle. In teleost fishes, however, there is little evidence concerning the rôle of the pituitary body. Houssay (1930, 1931) found that the injection of saline suspensions of the hypophyses from large fish (*Micropogon opercularis*) into small ones (*Cnesterodon*) was followed by the expulsion of eggs in from one to three days, although saline suspensions of muscle or saline solution alone produced no effect in control animals. This expulsion of eggs, however, preceded normal spawning by only 15 days. Cardoso (1934) injected saline suspensions of the pituitary bodies of *Pimelodus clarias* into other individuals of the same species. In both sexes he noted an increase in weight of the gonads over those of control animals, an effect much greater in the female. Pereira and Cardoso (1934), working with *Prochilodus*, injected saline suspensions of pituitary glands of the adult into females. In all cases ovulation occurred, varying from 24 to 96 hours after injection, although the injection of suspensions of nervous tissue into control animals did not result in ovulation. More recently von Ihering (1935) repeated these experiments, injecting suspensions of the pituitary glands of *Hopliius malabaricus* into two species of *Astyanax*. Mating activity was stimulated with an increase in size of both ovary and eggs over the untreated controls. When injections were made into *Prochilodus* of both sexes eggs and spermatozoa were obtained. Pituitary-like substances such as prolactin and extracts of the pituitary gland have likewise been used in fishes with some success. Calvet (1932) placed young *Petromyzon* in aquaria containing pregnancy urine and found that the ovary increased markedly in size over that of the controls. Damas (1933) injected 30 lampreys with pregnancy urine and obtained expulsion of eggs in all cases. He also employed extracts of the pars anterior of the pituitary gland with similar results. Boucher,

Boucher and Fontaine (1934) injected the urine of pregnancy into eels and reported enlargement of the gonads in both sexes and evidence of the maturation of spermatozoa in the testis. On the other hand, wholly negative results were obtained by Koch and Scheuring (1936) after injecting *Phoxinus* with both prolan and an extract of the anterior lobe of the pituitary gland.

EFFECT OF INJECTION OF PITUITARY EXTRACTS

In an effort to determine whether or not the pituitary gland of *Fundulus heteroclitus* is concerned in the control of the sex cycle two methods were employed, the injection of pituitary glands or extracts of them, and extirpation of the pituitary body. The results of the injection method have been disappointing. In male individuals no effect which can be ascribed to the material injected has been observed. The following is a brief summary of the results of several experiments. Following the successful use by Burns and Buyse (1933) of extracts of mammalian pituitary glands in stimulating precocious sexual development in *Amblystoma*, injections of a crude, alkaline extract of the whole pituitary gland of sheep were made into the body cavity of *Fundulus*.¹ In the first series records were obtained from 28 males so injected, with a number of uninjected controls. While spermatozoa were obtained in a number of cases they were also obtained in control animals, developing in both groups after the individuals had been maintained in the laboratory for some time. This appearance of spermatozoa was possibly a result of the change from the lower temperature of the Delaware river in which they were collected to the higher temperature of the laboratory. At any rate, that the appearance of spermatozoa in these males was not a result of the injections was shown by further experiments. Twenty-four males were divided, 12 placed in an aquarium in a constant temperature room at 5° C. and the other 12 kept in an aquarium in which the temperature of the water varied from 18° to 22° C. with an average temperature during the experiment of 20°. Since the constant temperature room was illuminated by daylight the light conditions in the two rooms were fairly comparable. Half the animals in each room were injected daily with 0.1 cc. of sheep pituitary extract. Records were obtained on 18 of these animals. In the warm room the testes were activated and spermatozoa appeared in both uninjected and injected animals in all cases after 7 days. In the cold room no spermatozoa appeared in either injected or in control animals. Microscopic examination of the testes showed that those of the injected animals were comparable to those of the uninjected controls, those of both groups in the

¹ I wish to express my thanks to Dr. Oliver Kamm of the Research Laboratories of Parke, Davis and Company for this material.

cold room showing less activity than was the case in the testes of animals from the warm room.

In females a more varied injection program was employed in an attempt to produce ripe eggs. The following materials were injected: whole pituitary extract of sheep, Antuitrin S (Parke-Davis), extracts of fish pituitary glands (*Fundulus* and *Mustelus*), and freshly ground pituitary glands of *Fundulus*. Records were obtained from 35 injected females. Only 4 of these, animals injected in March and early April, delivered ripe eggs. The earliest case of this occurred on March 16 after the animal had been in the laboratory for 11 days and had received 3 injections of 0.15 cc. each of sheep pituitary extract. The other 3 cases delivered eggs late in April (April 20-28) about one month before eggs were obtained from normal animals (May 27). While these 4 cases suggest the possibility that injection of pituitary extracts into *Fundulus* in early spring after the ovocytes have grown to a certain size may cause them to mature more rapidly than they normally do, certainly the number of cases is too small to be of much significance. In the 31 other cases no ripe eggs were obtained and microscopic examination of the ovaries showed no significant differences between them and those of control animals.

EFFECT OF HYPOPHYSECTOMY

The second method employed to study the influence of the pituitary gland on the gonad cycle in *Fundulus* was the removal of the gland. The operation was performed essentially in the manner previously described (Matthews, 1933) but with several modifications. A single, short mid-ventral incision was made in the branchiostegal membrane, reaching anteriorly as far as the base of the tongue. Through this incision the tongue was dissected free from the tissues of the floor of the mouth. The tip of the tongue was then pulled backwards and held at right angles to the ventral surface of the head with the left hand, its wedge shape serving to hold the wound open. After cutting through the mucous membrane in the roof of the mouth the parasphenoid bone was cut through on three sides, bent to one side of the median line to expose the hypophysis, and then replaced after the pituitary body had been removed. If the original incision in the branchiostegal membrane was of the proper length the tongue could be tucked through the incision and the free tip would hold it in place, thus closing the wound in the branchiostegal membrane without sutures. Healing in these cases was much more satisfactory than in the earlier operations.

This operation was carried out at two periods of the year. One group of animals was operated on in March and April, the other in October, November and December. The mortality, as usual, was high.

Of 176 operated animals weight records and fixed gonads were obtained from only 73,—33 males and 40 females. When the gonads from these animals were examined differences were noted between them and

TABLE I

Percentage of total body weight formed by the testis in hypophysectomized Fundulus as compared with normal and operated controls

Normal and Operated Controls			Hypophysectomized Animals		
Date Killed	Days Post-Operative	GW/BW (per cent)	Date Killed	Days Post-Operative	GW/BW (per cent)
NOR Oct. 10.		0.22	Oct. 19	10	0.21
Oct. 10.		0.31	Oct. 19	13	0.19
Oct. 10.		0.27	Oct. 26	13	0.22
			Oct. 24	18	0.26
			Oct. 26	20	0.19
			Oct. 28	21	0.25
HYC Nov. 17.	39	0.76	Nov. 7	32	0.21
			Nov. 27	48	0.37
HYC Dec. 3.	53	0.71	Dec. 3	51	0.33
HYC Dec. 3.	56	0.49	Dec. 17	66	0.27
			Dec. 24	73	0.43
HYC Mar. 12.	12	0.46	Mar. 7	5	0.46
			Apr. 20	5	1.05
			Mar. 11	9	0.77
HYC May 5.	206	1.20	May 4	10	1.48
HYC May 5.	206	2.01	May 6	12	0.81
			May 6	12	1.62
			May 6	13	0.43
			May 8	14	0.24
			May 10	16	0.38
			May 10	16	0.54
			May 12	18	0.29
			May 13	19	0.44
			May 7	22	0.67
			May 14	29	0.41
			May 16	31	0.21
HYC June 11.	48	1.26	June 11	48	0.30
NOR June 11.		1.96	June 11	49	0.28
			June 11	55	0.17
			June 11	55	0.08
			June 11	57	0.28
			June 11	57	0.17
			May 5	206	0.15

those of controls, particularly in the males. These changes were apparent in the weight of the testis relative to the total body weight, and in both gross and microscopic appearance.

Table I summarizes the way in which the testis weight changes after

hypophysectomy. As has been shown (Matthews, 1938) during the normal seasonal cycle the testis forms the smallest percentage of the body weight from September to December and the greatest in May and June. When the pituitary gland is removed in October there is no significant difference in the weight of the testes of experimental animals as compared with those of controls even after 73 days. In the one animal which survived until the next breeding season, however (206 days), the difference in the proportion of the body weight formed by the testis as compared with that of control animals was striking. Obviously the testis in this animal had not enlarged with the onset of the breeding season as did that of the control. In the series from which the pituitary gland was removed in March and April and the animals killed during the next breeding season in May when the testis normally reaches its greatest size, the same difference between the weight of the testes of control and of hypophysectomized animals was noted, though in these cases they had been hypophysectomized only 48 to 57 days previously. How soon after operation this difference in weight is noticeable cannot be accurately determined from this series due to an insufficient number of animals killed early in the experiment. It is clear, however, that there is no difference up to about 10-12 days after removing the hypophysis. After 13 or 14 days, however, the proportion of the total body weight formed by the testis is consistently and notably less than in control animals.

In gross appearance the differences between the testes of hypophysectomized and control animals are clear from Figs. 1 and 2. In place of the large whitish testis of the control animal that of the hypophysectomized individual is smaller and has the gray, translucent appearance of the testes of sexually inactive animals killed in late fall.

The microscopic appearance of the testes of these animals also shows differences between control and hypophysectomized individuals. Although the testes of animals operated on in October show no significant weight differences as compared with those of controls, the microscopic structure of these testes is different. The testes of control animals killed in October show a large number of primary spermatogonia, a number of secondary cells, including both secondary spermatogonia and primary and secondary spermatocytes which are multiplying to form cortical cysts, as well as a number of spermatids and a few spermatozoa (Fig. 3). In two animals killed during mid-October from which the pituitary glands had been removed 10 and 13 days previously the primary spermatogonia are as numerous as in the controls and are dividing in both cases as indicated by the presence of mitotic figures. The striking differences between these two testes and those of control animals

killed at the same time lie in the reduced number of cells of the second type and in the small number of mitotic figures in these as compared with those in the control testes (Fig. 4). In fact, the cysts, which are prominent in the control testes, are small and poorly defined in those from the hypophysectomized individuals. Moreover, spermatids and spermatozoa are extremely scanty. The testes of these two hypophysectomized animals present the picture of multiplication of spermatogonia without subsequent maturation of sperm cells. If this lack of maturation of spermatozoa is due to the lack of the pituitary gland, then it might be expected that such differences would be more marked in those hypophysectomized animals allowed to live on into the normal breeding season when maturation of sperm cells is most rapid. That such is the case is indicated in a number of such cases. Obviously the removal of the pituitary gland must precede the killing of the animal by a sufficient number of days for the effect on the testis to be noted. Thus in none of the animals hypophysectomized in March and April and killed from five to twenty-two days later are any differences observable in the testes of the operated and control animals. In fact, one of these animals killed on April 20, five days after removal of the pituitary gland, showed a definitely active testis (Fig. 5). The differences between this testis and that of one of the same series of operated animals killed on May 16 are well marked (Fig. 6). In the latter case the only cells present are primary spermatogonia and a few spermatids and spermatozoa. The differences between the two are such as might be explained by assuming that on April 15, when the pituitary glands were removed, rapid multiplication and maturation of the spermatogenic cells were well under way in preparation for the next breeding season. In the animal killed five days after removal of the hypophysis the lack of the gland had not as yet produced any effect on the testis. Twenty-six

EXPLANATION OF PLATE I

FIG. 1. Testis of control animal which had its pituitary gland exposed but not removed on October 10 and was killed May 5. $\times 1.4$.

FIG. 2. Testis of animal hypophysectomized on April 15 and killed June 11. $\times 1.4$.

FIG. 3. Cross-section of testis of normal animal killed October 10. $\times 194$. C, cortical cyst containing spermatocytes; PS, primary spermatogonia; S, spermatids.

FIG. 4. Cross-section of testis of animal hypophysectomized October 9 and killed October 19. $\times 194$. PS, primary spermatogonia; M, medulla of testis.

FIG. 5. Cross-section of testis of animal hypophysectomized April 15 and killed April 20. $\times 80$. C, cortical cysts containing cells which are dividing rapidly; SZ, spermatozoa.

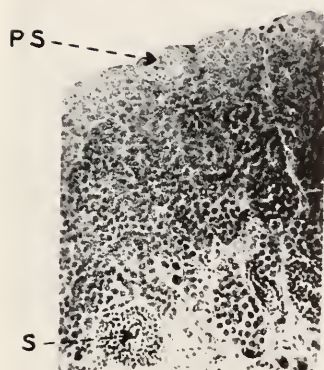
FIG. 6. Cross-section of testis of animal hypophysectomized April 15 and killed May 16. $\times 80$. PS, primary spermatogonia; SZ, spermatozoa.



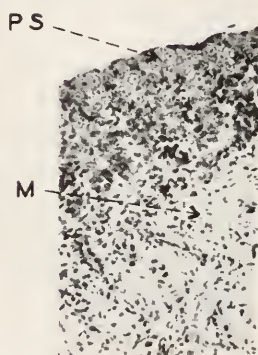
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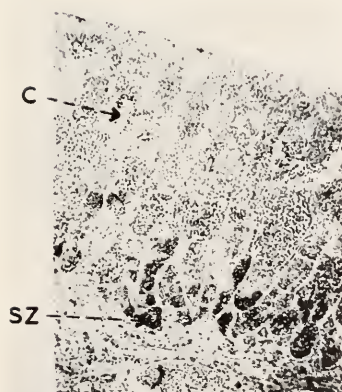
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5



6

days later, however, only a few spermatids and spermatozoa remain from the mass that had already been produced. Evidently after the lack of the pituitary gland has had time to manifest itself, further maturation of spermatogenic cells is either very much retarded or perhaps altogether lacking.

The most striking examples of this change in the testis following hypophysectomy were found in those animals which were allowed to run well over thirty days after removal of the pituitary gland and then killed close to or during the breeding season. Seven of these cases were observed with 5 control animals killed at the same time. These animals were killed from 48 to 206 days after removing the hypophysis.

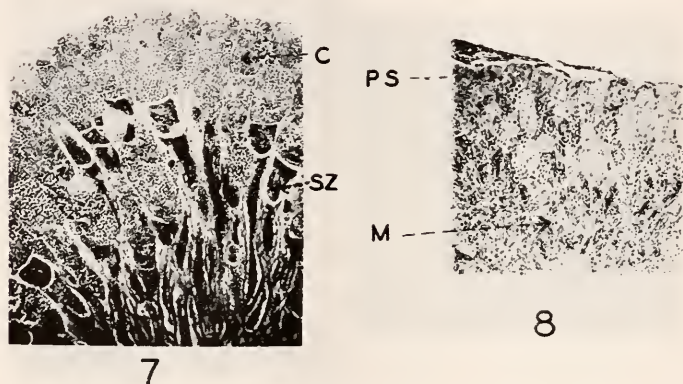


PLATE II

FIG. 7. Cross-section of testis of control animal which had its pituitary gland exposed but not removed on April 24 and was killed June 11. $\times 80$. C, cortical cysts with cells which are dividing rapidly; SZ, spermatozoa.

FIG. 8. Cross-section of testis of animal hypophysectomized April 24 and killed June 11. $\times 80$. PS, primary spermatogonia; M, medulla of testis.

With the exception of minor individual variations, differences in the microscopic appearance of the testes of these animals as compared with those of controls are much the same as those already described. The testis of all the hypophysectomized animals show only primary spermatogonia, a few scattered secondary spermatogonia and either very few spermatids and spermatozoa or none at all (Figs. 7 and 8). It might be noted, however, that in all these animals the primary spermatogonia are dividing, even in the one case from which the pituitary gland had been removed 206 days previously.

The data for hypophysectomized females are at present very scanty. As in the male, there is little change in weight of the female gonad in

those cases killed in the fall months and differences in the microscopic structure of the ovary of hypophysectomized as compared with control animals are hard to detect. Although more females were operated on than males the mortality of these animals was particularly high as the breeding season approached. As a result records on hypophysectomized females killed during May and June have been obtained on only a few animals. The oldest of these in post-operative age was killed only 55 days after removal of the hypophysis and records were obtained on only two others which were killed more than 30 days following hypophysectomy. In all three cases, however, the ovary was lighter relative to the total body weight than in control animals, several of which ran 206 days after exposing but not removing the pituitary gland. An example is provided in case HY 4/17-6/11 in which the ovary formed 1.52 per cent of the body weight 55 days after removing the hypophysis as compared with HYC 10/10-5/5 (206 days post-operative, 4 individuals) in which the average weight of the ovary constituted 2.87 per cent of the body weight. It is obvious, however, that more cases from which the hypophysis has been removed for more than 30 days prior to the normal breeding season must be obtained before the effect of the loss of the pituitary gland on the ovary may be accurately determined.

SUMMARY

Injections of pituitary extracts into male *Fundulus* have been practically without stimulating effect on the testis. In females records were obtained from thirty-five cases of which only four delivered eggs earlier than did control animals, one case delivering ripe eggs six weeks, the other three cases about four weeks before the normal breeding season.

After removing the pituitary gland the gonads undergo regressive changes as compared with the controls, changes which are particularly striking in the testis. When the pituitary gland was removed in the fall and the animals were allowed to run until the next breeding season in May and June the testes failed to enlarge as they normally do. Microscopically such testes showed numerous primary spermatogonia but very few spermatocytes and practically no spermatozoa. When the pituitary gland was removed in March and April, after the testis had already enlarged somewhat, fourteen to twenty-one days after operation the testes had become notably smaller than in control animals, and showed only primary spermatogonia with a few spermatids and spermatozoa. These results indicate that the pituitary gland exerts a controlling influence on the seasonal cycle which the testis of this teleost fish exhibits. This influence is apparently of greater importance in maturation than in proliferation of the germ cells.

LITERATURE CITED

- BOUCHER, S., M. BOUCHER, AND M. FONTAINE, 1934. Sur la maturation provoquée des organes génitaux de l'anguille. *Compt. Rend. Soc. Biol.*, **116**: 1284.
- BURNS, R. K., AND A. BUYSE, 1933. The induction of precocious maturity in the reproductive tract of recently metamorphosed female salamanders, by an extract of the mammalian hypophysis. *Anat. Rec.*, **58**: 37.
- CALVET, J., 1932. Action du lobe antérieur d'hypophyse chez divers Vertébrés (Lamproies, Oiseaux). *Compt. Rend. Soc. Biol.*, **109**: 595.
- CARDOSO, D. M., 1934a. Relations entre l'hypophyse et les organes sexuels chez les poissons. *Compt. Rend. Soc. Biol.*, **115**: 1347.
- CARDOSO, D., 1934b. Relação genito-hipofisaria e reprodução nos peixes. *Archiv. Instit. Biol.*, **5**: 133.
- DAMAS, H., 1933. Note sur l'apparition naturelle et provoquée des caractères sexuels chez la lamproie. *Bull. Soc. Roy. des Sci. de Liège*. No. 4, p. 94.
- HOUSSAY, B. A., 1930. Acción sexual de la hipofisis en los peces y reptiles. *Revista Soc. Argentina Biol.*, **6**: 686.
- HOUSSAY, B. A., 1931. Action sexuelle de l'hypophyse sur les poissons et les reptiles. *Compt. Rend. Soc. Biol.*, **106**: 377.
- VON IHERING, R., 1935. Die Wirkung von Hypophyseninjektion auf den Laichakt von Fischen. *Zool. Anzeig.*, **111**: 273.
- KOCH, W., AND L. SCHEURING, 1936. Die Wirkung von Hypophysenvorderlappenhormon auf den Laichakt von Fischen. *Zool. Anzeig.*, **116**: 62.
- MATTHEWS, S. A., 1933. Color changes in *Fundulus* after hypophysectomy. *Biol. Bull.*, **64**: 315.
- MATTHEWS, S. A., 1938. The seasonal cycle in the gonads of *Fundulus*. *Biol. Bull.*, **75**: 66.
- PEREIRA, J., JR., AND D. M. CARDOSO, 1934. Hypophyse et ovulation chez les poissons. *Compt. Rend. Soc. Biol.*, **116**: 1133.

STUDIES ON VIRUS DISEASES OF FISH

II. LYMPHOCYSTIS DISEASE OF *FUNDULUS HETEROCLITUS*¹

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At the meeting of the American Society of Zoologists at Indianapolis, December 1937, the author presented a paper and a demonstration concerning "intracellular parasitism in fish producing a gigantic growth of the infected cells."³ This paper also dealt with parasitic protozoa as well as with the lymphocystis virus disease and the peculiar structure of the lymphocystis cells. With reference to this latter, Dr. Roland Walker, Biological Laboratories, Rensselaer Polytechnic Institute, Troy, New York, sent to the author sections through a tumor of the common salt water killifish *Fundulus heteroclitus*, which he had preserved in August, 1937, at the Marine Biological Laboratory, Woods Hole, supposing that this tumor represented something similar to the demonstrated hypertrophied fish cells.

Studying these preparations I could easily verify that the tumor indeed represents a lymphocystis tumor, never described hitherto on *Fundulus heteroclitus*. I am greatly indebted to Dr. Walker, who decided to leave the description of this interesting observation entirely to me.

The significance of this discovery is not represented so much by the finding of a new host of lymphocystis disease, but by the fact that this fish is known to be easily kept under laboratory conditions. Therefore, it can be hoped that lymphocystis disease could be cultivated in the laboratory for further experimental studies if sufficient attention were paid to a new appearance of this virus disease on *Fundulus heteroclitus*.

The writer estimates from the series of sections that the tumor probably had a diameter of 2 to 3 mm. The localization of the tumor was in the membrane of the tail fin. The preserved fish was the only specimen among 200 *Fundulus heteroclitus* on which Dr. Walker ob-

¹ Studies on virus diseases of fish. I. Lymphocystis disease of the Orange filefish (*Aleutera schoepfii*) appeared in the *Amer. Jour. Hygiene*, Vol. 28, No. 3, 455-462, November, 1938.

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³ Abstract in *Anat. Rec.*, 70: No. 1, Suppl. No. 1, 68.

served such a tumor. The tumor, preserved with Bouin's fluid and stained with Harris' hematoxylin, consists of large cells of diameters of 135 to 225 microns, located in the connective tissue of the fin membrane.

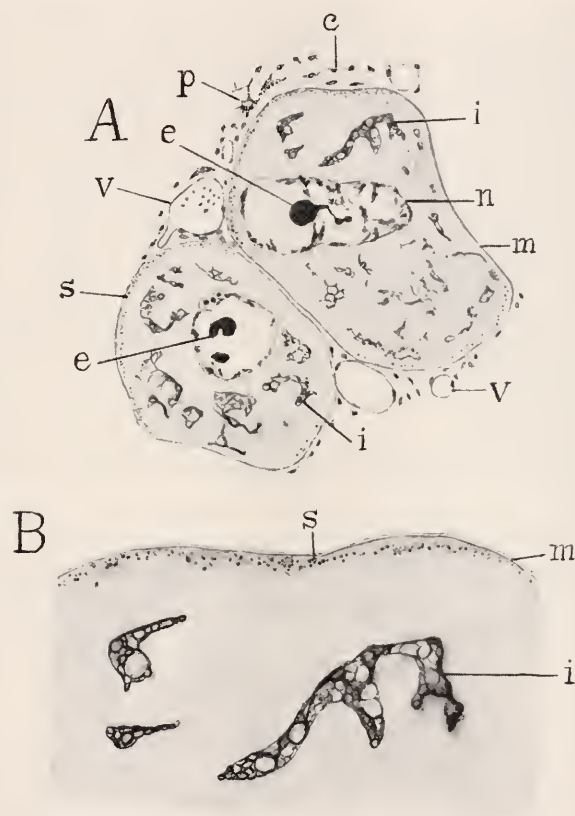


FIG. 1. *A*. Two lymphocystis cells of *Fundulus heteroclitus*. $\times 210$. *B*. Upper part of the upper cell with higher magnification. $\times 600$. *n*, nucleus; *e*, nucleolus; *i*, inclusion bodies; *m*, membrane; *s*, strongly stained granules in the cortex of the lymphocystis cells; *c*, small connective tissue cells; *p*, pigment cell; *v*, vessels.

These large cells (Fig. 1*A*) contain in their cytoplasm the peculiar reticular inclusion bodies which represent the most conspicuous structural element of lymphocystis cells. The inclusions (*i*) appear in the sections as separate coils, but are probably part of a continuous network, similar to those found in the lymphocystis cells of the perches

(*Accerina*, *Stizostedion*). Figure 1*B* shows with higher magnification their basophilic framework representing a reticular or alveolar structure, strongly stained with hematoxylin. The meshes or alveoles are filled with a paler tingible substance.

These inclusion bodies are so characteristic that the diagnosis of lymphocystis cells is assured without further ado although the second typical structure of the lymphocystis cells, the cell-membrane, shows a remarkably poor development. The cell-membrane appears in Fig. 1*A*

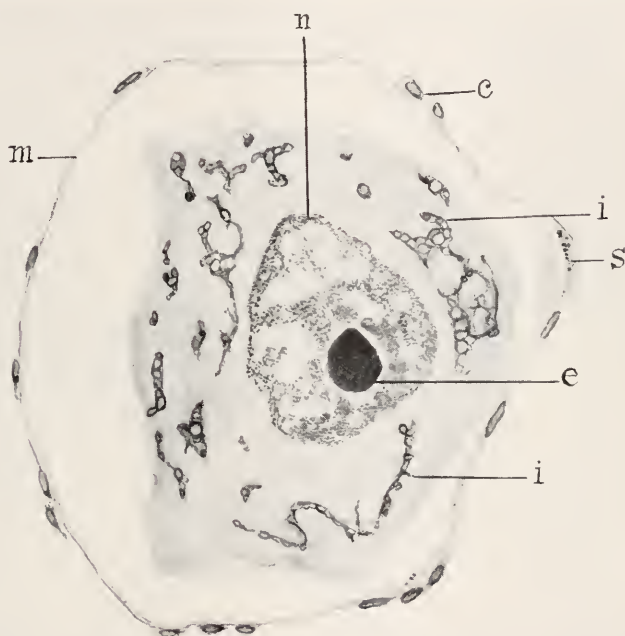


FIG. 2. Lymphocystis cell of *Fundulus heteroclitus*, the cytoplasm of which has withdrawn by shrinking from the membrane (*m*). *n*, nucleus; *e*, nucleolus; *i*, inclusion bodies; *s*, strongly stained granules in the cortex of the lymphocystis cell; *c*, small connective tissue cells. $\times 600$.

only as a contour (*m*) and also with higher magnification (Fig. 1*B*), the configuration of the membrane is not very clear. But cells, in which the cytoplasm has withdrawn from the membrane by shrinking (Fig. 2), show distinctly that the membrane is represented by a cuticula of double contour, evidently homologous to the thicker membranes of the lymphocystis cells of other fish.

The nuclei contain in their peripheral zone a fairly large amount of chromatic substance as bars and fields of granules. One or two nucleoli

(*c*) are to be seen which, as it seems, can give origin to buds (Fig. 1*A*). Just beneath the membrane of the lymphocystis cells a layer of irregular granules (*s*), strongly stained with hematoxylin, could be observed, which, as a rule (Fig. 1*A*), have only developed on those planes of the cells which face the surfaces of the fin membrane. Something similar was seen by the author hitherto only on some degenerating lymphocystis cells of *Pleuronectes flesus*, but there in the whole peripheral zone of the cells.

Because it would be very important for the further research on lymphocystis virus to obtain a stock of lymphocystis-affected fish which might be easily kept in aquaria, the writer attempted to pursue in 1938 the trail found by Dr. Walker. Unfortunately, it seems that the occurrence of lymphocystis disease on *Fundulus heteroclitus* is very rare. Five hundred and fifty specimens of this species, preserved at the Marine Biological Laboratory, Woods Hole, in August 1938, were thoroughly examined without discovering any other case of lymphocystis disease.

Further, there was no success in attempting to transmit to *Fundulus heteroclitus* the lymphocystis disease of the wall-eyed pike perch, *Stizostedion vitreum*, fresh material of which could be procured from the Great Lakes. Corresponding experiments to transmit the *Stizostedion*-disease to the freshwater killifish, *Fundulus diaphanus*, gave the same negative result. The method applied in these experiments consisted in dispersing an emulsion of *Stizostedion*-lymphocystis tumors into the aquarium and in feeding small pieces of the tumors. The *Fundulus*-fish of both species were very eager to swallow the *Stizostedion*-lymphocystis cells, but none of 16 *F. heteroclitus* and of 12 *F. diaphanus* became infected.

Concerning this result, the following facts may be taken into account: (1) The transmission of lymphocystis disease under laboratory conditions was hitherto only successful by keeping diseased and healthy fish together or by the method applied in the *Stizostedion*-*Fundulus*-experiments which attempted to imitate the natural conditions of infection. (2) This method gave, as a rule, positive results in high percentage in all experiments in which the applied tumor material originated in the same kind of fish to which the treated fish belonged. [See experiments of Weissenberg in Europe on *Acerina cernua* (1914)⁴ and *Pleuronectes flesus* (1921)⁵ and in this country on *Stizostedion*

⁴ Weissenberg, R., 1914. Über infectiöse Zellhypertrophie bei Fischen (Lymphocystiserkrankung). *Sitzungsber. d. Kgl. preuss. Akad. d. Wiss., Physik. mathem. Kl.*, 30: 792-804.

⁵ Weissenberg, R., 1921. Lymphocystiskrankheit der Fische. *Handbuch der Pathogenen Protozoen herausgegeben von v. Provasch-Nöllner*, 3: 1344-1380.

vitreum (still unpublished)]. (3) The lymphocystis disease of *Acerina cernua* was discovered by Weissenberg on a Baltic Sea tribe of this species. A freshwater tribe of the same species showed in an infection experiment a lower susceptibility than the original tribe (Weissenberg, 1914, l.c., p. 802). (4) Experiments to infect fishes of other species, genera or families by keeping them together with lymphocystis-affected fish has hitherto always given negative results. But these observations (Joseph, 1918; ⁶ Weissenberg, 1921, l.c., p. 1348) referred to fish on which lymphocystis disease is not observed in nature.

The negative result in the aforesaid experiments to transmit lymphocystis disease from *Stizostedion vitreum* to *Fundulus heteroclitus* and *diaphanus*, in which experiments a certain susceptibility for the disease could be supposed at least for *F. heteroclitus* on account of the described case of Woods Hole, is in accordance with the interpretation that different kinds of lymphocystis viruses are to be distinguished which are adapted to different fishes.

It would be very desirable to pay attention to further cases of lymphocystis disease of *Fundulus heteroclitus* and to keep affected specimens together with healthy fish of this species for cultivating a stock of lymphocystis-affected fish, which could easily be kept under laboratory conditions and therefore would be well adapted for the further study of this interesting virus disease in this country.

SUMMARY

The first and until now solitary case of lymphocystis disease in *Fundulus heteroclitus* is described. *Fundulus heteroclitus* and *F. diaphanus* proved not susceptible to the virus of the lymphocystis disease of *Stizostedion vitreum* in infection experiments.

⁶ Joseph, H., 1918. Untersuchungen über Lymphocystis Woodc. *Arch. f. Protist.*, Bd. 38.

DIURNAL VERTICAL MIGRATIONS OF DEEP-WATER PLANKTON

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INTRODUCTION

Diurnal vertical migrations have long been known to play an important part in the lives of pelagic organisms. The numerous previous studies on these migrations in oceanic animals have been limited almost entirely to plankton living relatively near the surface. It was the purpose of the present investigation to continue and extend the observations made by Welsh, Chace, and Nunnemacher (1937) on the diurnal vertical migrations of deep-water animals. More specifically, our objectives were (1) to ascertain the vertical extent of these migrations for the bathyp plankton,² (2) to determine the greatest depth in the sea at which these migrations still occur, and (3) to discover the relations of these phenomena to the changes in submarine illumination with time and depth.

HISTORICAL CONSIDERATIONS

The earliest knowledge of diurnal vertical migrations of marine organisms arose from the practical observations of fishermen. Thus man has known from time immemorial that under certain conditions if he wishes to obtain a good catch of herring, his nets should be set deep in the water during the daytime and shallower at night. Such observations were abundantly confirmed and extended for shallow-water animals by the work of a great many marine biologists (an extensive literature is cited by Rose, 1925; Russell, 1927; and Clarke, 1933b).

As far as the plankton of deeper water was concerned, a small amount of information was also already at hand by quite an early date. For example Rang (1828) observed that the pteropod, *Hyalocylis striata*, among others, could be taken at the surface of the sea only after sunset and before sunrise and sank into deeper water during the day. Recently Stubbings (1938) has found that this species descends during

¹ Contribution No. 205, Woods Hole Oceanographic Institution.

² See Ekman ('35, pp. 451-77) for a comprehensive general discussion of the bathypelagic fauna.

the day to 200–250 m. so that for at least part of the day it is a bathypelagic organism, i.e., one which lives in the intermediate depths rather than near the surface or the bottom of the sea. But the significance of this and other similar early observations was not appreciated until long afterwards since the existence of a bathyplankton had not been realized until the latter part of the nineteenth century when the data of the various great oceanographic expeditions were analyzed.

Murray (1885, p. 218) concluded from his experiences on the "Challenger" that, "the great majority of plankton organisms live at various depths down to and even deeper than 100 fathoms during the day . . . and only come to the surface at night. . . ." Other investigators came to similar general conclusions (e.g., Chun, 1890, pp. 82–4; Brauer, 1906, p. 337; 1908, p. 232). Brauer was among the first to point out the possible relation of these migrations to such interesting biological problems as bioluminescence, type and size of eye, and pigmentation in the bathyplankton.³ However, it remained for Fowler (1905, 1909) first to demonstrate by means of numerical data for schizopod and ostracod crustaceans the diurnal vertical migrations of moderately deep-living species. Soon afterwards Murray and Hjort (1912) showed clearly that such migrations were undertaken by organisms which were definitely "deep-sea" animals during the daytime; these investigators also emphasized the correlation of these phenomena with the general biology of the sea, sustaining and extending some of Brauer's suggestions by means of their more complete data.

The further inquiry into the exact and quantitative aspects of the subject presented considerable technical difficulties since for this purpose it is necessary to know accurately where the plankton has been caught, which is impossible when working with open nets. Hence the precise determination of the vertical extent of diurnal migrations and of the various depths at which they occur had to await the development of efficient deep-sea equipment for sampling populations at known depths without contamination from other strata.

Since the invention of the Sigsbee gravitating deep-sea trap (1880) and Palumbo's closing net (Chierchia, 1885, p. 81), many oceanographers have devised such apparatus; yet it was only very recently that reliable equipment has been available for these purposes.

METHODS

The observations were carried out in July, 1937, at "Atlantis" Station 2894 (39° 06' N., 70° 16' W.) which is in continental slope

³ For recent investigations of these problems see Welsh and Chace, 1937, 1938.

water about 300 miles east of Cape May, N. J. This position was chosen for several reasons: (1) The slope water, except for the superficial layers, is relatively free from currents (see Iselin, 1936, p. 11). (2) The station was far enough from both the Gulf Stream and coastal waters to be free of their influence. (3) The water was sufficiently deep to prevent benthonic organisms from confusing the results. (4) Data from the continental side of the Gulf Stream might offer some interesting points for comparison with results previously obtained (Welsh, Chace, and Nunnemacher, 1937) in the far more transparent waters of the Sargasso Sea.

In order to keep the ship in about the same geographic position the hauls were made in various directions while the work was being done. The maximum deviation from the original station was about 14 miles (see Table I).

TABLE I

Position of the ship at various times while the work was being carried out. The first position in the table is that designated as Station 2894.

Date	Time	Latitude	Longitude
July 18th.....	10 P.M.	39°06' N.	70°16' W.
19th.....	5 A.M.	38°57'	70°30'
19th.....	Noon	39°01'	70°30'
20th.....	Noon	39°06'	70°33'
21st.....	Noon	39°00'	70°30'
22nd.....	Noon	38°59'	70°30'

Horizontally hauled closing nets were used, modified from those employed by Leavitt (1935, 1938) as described by Welsh, Chace, and Nunnemacher (1937). These were 2 m. in diameter at the mouth and 9 m. long; the sides were of stramin, 6 threads to the centimeter, with a silk inset, about 20 threads to the centimeter (54 to the inch), in the tail. While the nets were open, the speed of the ship relative to the surface water was kept close to 2 knots. However, the actual speed of each net in relation to the water layer through which it was moving was not known. Since the water at various depths was probably moving at different velocities and possibly even in different directions, the results in such a situation might be very misleading as to the actual population distributions. But in the present case the fact that the hauls were made in various directions precludes the origin of systematic errors from this source. Furthermore, the evidence from the catches for a regular diurnal migration in the various organisms indicates that the relative

movements of the water were not of sufficient magnitude to confuse the results.

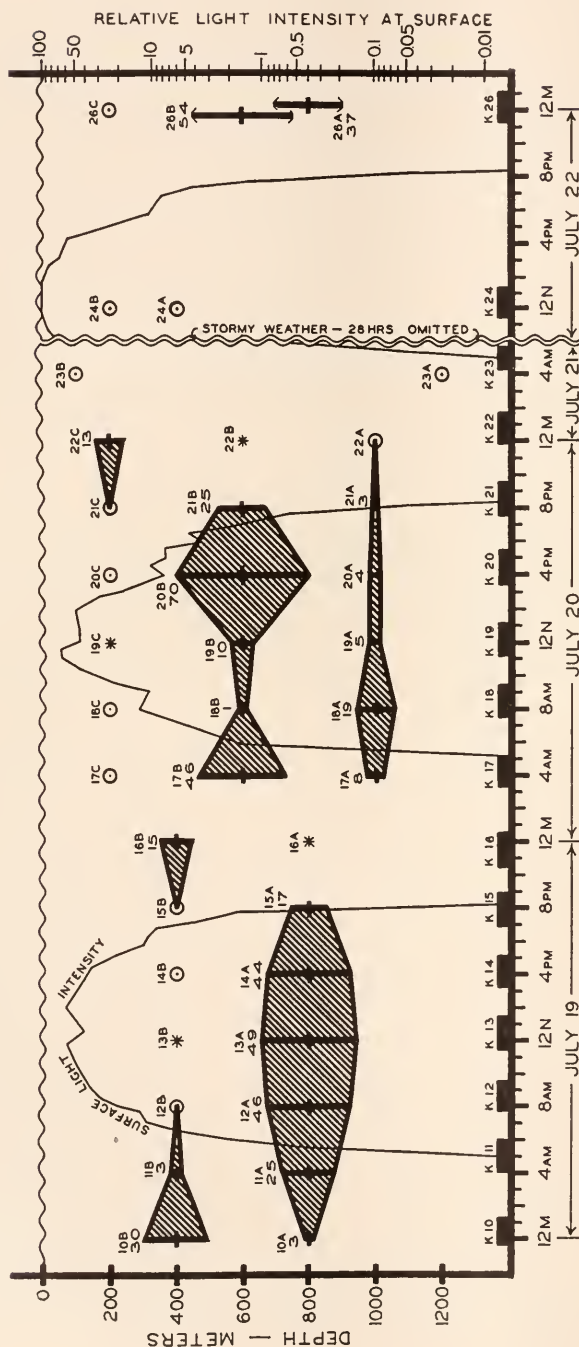
The depth at which a net was fishing was estimated by means of the trigonometric relation between wire angle and the amount of cable out. At 1,000 m. this estimate was probably accurate within about 10 per cent, being more accurate in shallower and less so in deeper tows.

Thirty-nine catches in a series of 16 hauls were made with the nets fishing for 2 hours at various depths and times throughout more than 2 diurnal cycles. Each entire haul took 4 hours so that 6 were made in a 24-hour period. Tows were made at 100, 200, 400, 600, 800, 1,000 and 1,200 meters, calculated depth, and since the bottom was at 2,860 m., the population thus sampled was purely pelagic. Although the closing nets worked very well in general, 4 of the 39 hauls failed; these are marked in Fig. 1 by asterisks.

Figure 1 represents graphically the times and depths of all the closing net hauls made at this station and shows that various depths were sampled on succeeding days. During the morning of the third day on station a series of tows at 100 and 1,200 m. was begun, but the sea became too rough to continue towing (force 5 on the Beaufort scale) so only one haul was made before work was stopped. In an attempt to fill in the gaps in our series we made tows at appropriate depths at noon and midnight on July 22, i.e., about 36 and 48 hours after the continuous series of hauls had been abandoned.

Throughout the periods when the plankton hauls were being made, a record was kept of the light intensity on deck; furthermore, two series of measurements of submarine illumination were carried out. The same submarine and deck photometer cases were employed and the same procedure followed as previously described by Clarke (1933a, 1938a).⁴ However, the Westinghouse "photox" cells in his apparatus were replaced by emission photoelectric cells of the "photronic" type (manufactured by the Weston Electrical Instrument Corp.) since the former had been found to be somewhat variable. To measure temperature changes, a small thermometer was installed inside the case of the deck photometer, beside the cell, where it could be read through the photometer window by raising the opal glass momentarily. The temperature inside the photometer case during the day was found to vary between a minimum of 19° C. and a maximum of 35° C. Correction factors throughout this temperature range were determined from tests carried out in the laboratory at the end of the cruise, in collaboration with

⁴ In making these measurements the shadow of the hull is avoided by heaving the ship to with her stern toward the sun and suspending the sea photometer from the end of the mizzen boom (compare criticism by Poole, 1938).



Dr. W. R. Sawyer. The proper correction, varying up to a maximum of 12½ per cent, has been applied to each of the photometer readings.

In the first series of measurements of the penetration of light into the water (Series 450) the photronic cell was used with opal diffusing disc, but no filter. Thus the spectral sensitivity of the photometer was that of the photronic cell itself (See Weston Electrical Instrument Corp. Circ. TD-10), the sensitivity of which reached a maximum at 5800 Å. and dropped off to 10 per cent of this value at 7080 Å. and 3420 Å. In the second series (Series 451) the "green" filter combination (Schott-Jena BG-18 and GG-11) used previously and the opal disc were employed. The transmission of this green filter combination exhibited a maximum at about 5100 Å. and fell to 10 per cent of this value at about 6460 Å. and 4640 Å. When these filters were used their selective action was superimposed upon that of the photronic cell with the result that the point of maximum sensitivity of the whole instrument fell between 5800 Å. and 5100 Å.

RESULTS

This paper presents the results on the occurrence and diurnal vertical migrations of the eleven most numerous malacostracan crustaceans. The decapod, *Gennadas elegans* (Smith), has been chosen for detailed analysis of the data since its presence in significant numbers (526 specimens), its apparent freedom from marked swarming, and its definite diurnal migrations make it a favorable form for our purpose. This prawn was taken at all depths except 100 and 1,200 m. although 83 per cent of the adults captured occurred at 600 and 800 m.

Figure 1 shows that during the first day the number of *Gennadas* present at 800 m. showed a 15-fold increase between midnight and 8 A.M., followed by a further small increase by noon. By 4 P.M. the population present at this depth had decreased to 35 per cent of the number there at noon. Since *Gennadas* disappeared entirely from 400 m. or shallower after 4 A.M. and was not found at this depth again until midnight, the catches strongly suggest that individuals appearing at 800 m. between 4 A.M. and noon had migrated down from lesser depths. The series of hauls at 200, 600, and 1,000 m. on the second day confirmed this indication by showing that the *Gennadas* stock which was present at 200 m. at midnight moved downward a considerable distance during the morning and returned again to shallow water during the afternoon and evening. The numbers taken at 600 m. at various hours of the day suggest further that the bulk of the *Gennadas* stock has gone below 600 m. at the deepest extent of this vertical migration. It is noteworthy that the only hour at which these crus-

taceans were taken at 200 m. was midnight when a significant number of them were taken at that depth. Clearly a shifting of the population of this species by nearly 400 m. took place on both days when hauls were made.

The conclusions drawn from the above horizontal histograms (Fig. 1) appear even more clearly when the results of the different days are combined and regraphed in the conventional vertical histograms (Fig. 2). It is furthermore apparent from this graph that the vertical spread of the population remained about the same throughout the 24 hours in spite of its bodily translation up and down. Hence these migrations involved a concerted vertical movement of the population as a whole rather than massing and scattering effects due to relative movements between various parts of it.

One of the more striking features of the behavior of the *Gennadas* population shown in Fig. 2 is that the animals were apparently not at the same depths at each midnight. Examination of Fig. 2 shows that the *Gennadas* as a whole are considerably deeper in the water in the second midnight histogram than they are in that for the first midnight. Reference to Fig. 1 demonstrates that the difference between these two histograms depends on the deeper distribution of this prawn on the midnight following the storm of the twenty-first than at the same hour on the preceding days. Specifically, on the midnight of the fourth day none were taken at 200 m. in contrast to the 13 taken there two days before, whereas twelve times as many were taken on this occasion at 800 m. as were taken at the same time and depth four days before.

Our data yield some further details concerning the behavior of *Gennadas elegans*. There were no significant differences in vertical distribution and migration between the males and females (adult). On the other hand, there were notable differences appearing between the behavior of the adult and the post-larval specimens. All of the 67 post-larvae of *Gennadas* taken in our hauls were caught at 600 m.; none appeared at any other depths. Thus, if the post-larvae underwent diurnal migrations, the vertical extent of these was not great enough to bring the animals at any time, in sufficient quantities to be caught, within strata sampled by the other nets.

The evidence at hand for *Hymenodora glacialis* (Buchholz) is unfortunately more circumstantial than conclusive (Fig. 3A). Adults were taken only at 1,000 m. and in small numbers; from these facts and from the results of previous work (see Welsh and Chace, 1937, p. 63) it is clear that the adults of *Hymenodora* live generally deeper in the water than our nets fished. The post-larval and immature specimens of this crustacean were living shallower so that a considerable

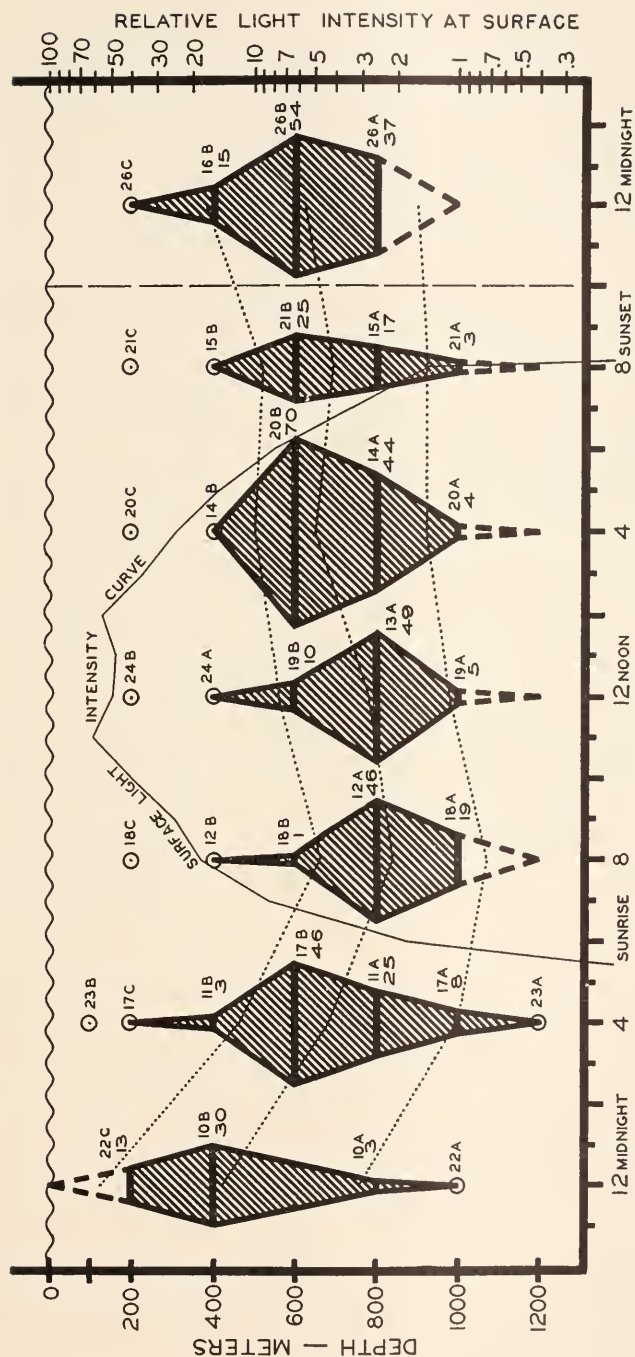


FIG. 2. *Gennadas elegans*: distribution of the adults in the various catches so arranged as to represent the position of the population at the different hours of the day. The small numerals (22C, 10B, etc.) to the right of the histograms are the numbers of the nets, which are given so that the method of combining the data of the different days will be clear from a comparison with Fig. 1. The vertical dashed line separating the second midnight histogram from the rest emphasizes the fact that 3 of the 4 nets in the group were hauled after the storm of July 21. The horizontal bars of the histograms and the larger numerals represent the numbers of animals. The upper and lower dotted lines delimit boundaries of 80 per cent of the population and the middle dotted line represents its center. The light intensity curve is an average for the 4 days measured.

number of them was captured. Sixty per cent of the 153 specimens in the hauls were taken at 1,000 m. and none were caught at any time of day above 800 m. It is unfortunate that the 600 m. net on the midnight of July 20-21 failed to fish so that we do not know whether this prawn was present at this crucial point or not. If we assume on the basis of evidence afforded by some of the other crustaceans, as we have done above for *Gennadas*, that the population was about 200 m. lower in the water on July 22 than on the days preceding the storm of July 21, we can conclude that *Hymenodora* probably was present at 600 m. on the midnight of July 20-21. Owing to the lack of hauls deeper than 1,000 m. during the middle of the day, the lower boundaries of five out of the seven histograms are indeterminate. Indecisive as the present data may be, it is probable, nevertheless, that this animal was undertaking diurnal vertical migrations of about 200 m. amplitude.

Parapasiphaë sulcatifrons Smith (Fig. 3B) was the next most numerous decapod crustacean in our catches after *Gennadas* and *Hymenodora*. Fifty-nine per cent of the 123 specimens⁵ taken occurred at 800 m. and 10 per cent of them at 1,000 m. Furthermore, this prawn was never caught at 200 m. at any time of day and was taken at 400 m. only at midnight. Thus the general vertical distribution of this species was appreciably deeper in the water than that of *Gennadas* but not of *Hymenodora*.

A diurnal vertical migration of *Parapasiphaë* also occurred. The downward movement was essentially accomplished in the four hours from midnight to 4 A.M. and the upward migration apparently started slowly in the late afternoon although 75 per cent of its extent was accomplished more rapidly after 8 P.M. The fact that these animals, too, were considerably lower in the water on July 22 is shown by the differences between the histograms for the first and second midnights in Fig. 3B.

Only one of the six species of *Sergestes*, *S. arcticus* Kröyer, taken at this station occurred in sufficient numbers for our present purpose. An examination of the distribution in the catches of the 104 specimens of carapace length of 10 mm. or over (Fig. 3C) shows that the older stages of this prawn were living shallower in the water than either *Parapasiphaë* or *Gennadas* since the center of the average vertical distribution of the population was just above 600 m. rather than about 800 m. as it was for the other two forms. These *Sergestes*, moreover,

⁵ Immature, post-larval, and larval specimens were included in this count as well as in the figure. Although the immature specimens were not identified as to species, they were included here as *P. sulcatifrons* since all individuals whose development was sufficiently advanced to permit identification belonged to this species.

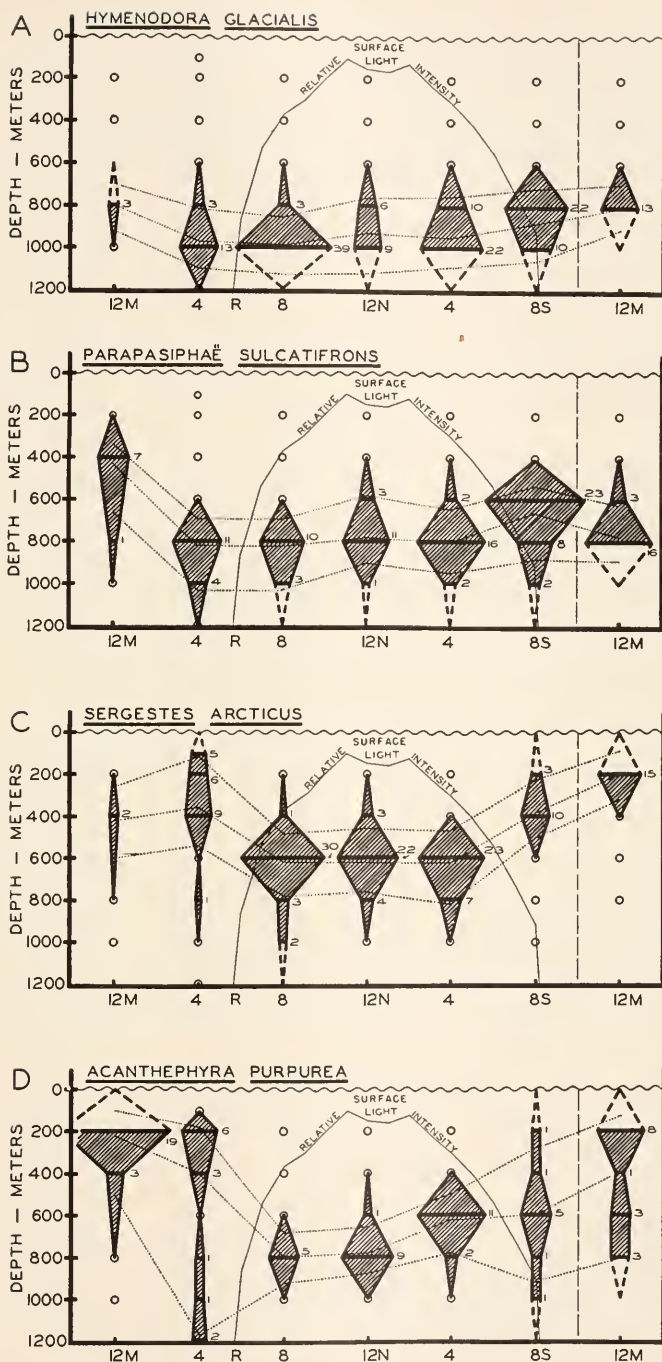


FIG. 3. Decapods: data condensed as in Fig. 2. The numerals to the right of the histograms represent the numbers of specimens taken. These figures include: *A*, larval, post-larval, and immature stages; *B*, all stages; *C* and *D*, adults.

also exhibited an extensive diurnal vertical migration of at least 200 and probably as much as 400 m.

The absence of significant numbers of the species in the hauls made for the first midnight of Fig. 3C was probably due to the fact that most of the population was in less than 200 m. of water at this time. Some circumstantial support is given this explanation by the presence of some of these decapods at 100 m. at 4 A.M. and by the position of the population shown by the histogram of the second midnight in Fig. 3C since the stock of *Sergestes* by analogy with observations on several of the other Crustacea would be lower in the water by about 200 m. than it would have been at the same time under the conditions of the preceding days.

There is some evidence from an analysis of the catches that those *Sergestes arcticus* of carapace length less than 10 mm. live at and migrate to slightly shallower levels than the larger individuals shown in the figure and discussed above, but hauls closer together than ours would have been necessary to determine this relation quantitatively.

Only 68 *AcanthePHYRA purpurea* A. Milne-Edwards (Fig. 3D) were taken in our hauls, and these had a rather patchy distribution. Nevertheless, there is some definite evidence for the occurrence of a diurnal vertical migration of at least 400 and possibly 600 m.

Among the more primitive malacostracans two species of euphausiids, two species of mysids, and two species of amphipods were caught in sufficient numbers to provide adequate samples for the present analysis.

The relatively enormous number of 180,000 specimens of the euphausiid, *Nematoscelis megalops* G. O. Sars (Fig. 4A), was taken. The 1,543 adults among these were living mainly between 400 m. and the surface although 12 of them were taken in the only 1,200 m. haul. From the evidence of the very patchy occurrence of all the *Nematoscelis*, one would conclude that this species swarms markedly, which may account for the extremely small numbers caught in the noon hauls. Clearly, however, a diurnal vertical migration at least 200 m. in extent was being accomplished by the adult *Nematoscelis*, shown in the figure.

The other schizopods taken in significant numbers were also migrating vertically, as much perhaps as 600 m. in the case of *Thysanopoda acutifrons* Holt and Tattersall (Fig. 4B), and 400 m. in those of the mysids, *Boreomysis microps* G. O. Sars (Fig. 4C), and *Eucopia unguiculata* (Willemoës-Suhm) (Fig. 4D). A comparison of the histograms of the first and second midnights in Figs. 4B, C, and D, indicates that these organisms, as well as several of the previously discussed Crustacea, were 200 m. deeper in the water after the storm of July 21

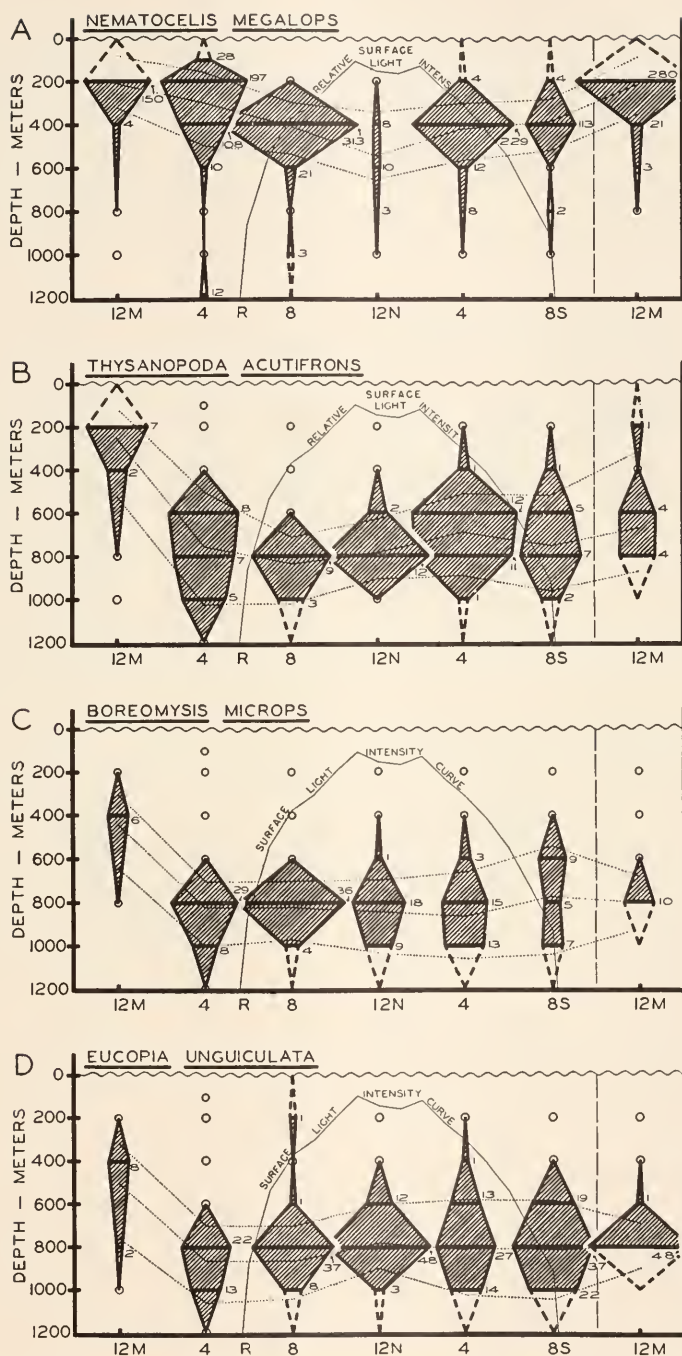


FIG. 4. Euphausiids and mysids. A, large adults; B and C, adults; D, all stages. See Fig. 2 for further explanation.

than they were on the previous two days. It is furthermore noteworthy that the *Thysanopoda* (Fig. 4B), as well as the *Gennadas* (Fig. 2), *Parapasiphaë* (Fig. 3A), and *Acantheephyra* (Fig. 3D) apparently began to move slowly upwards in the water almost as soon as they had reached the lowest point of its downward excursion in the morning; this slow upward migration was succeeded at about 8 P.M. by a more rapid movement which brought the animal to the highest point of its migration about midnight.

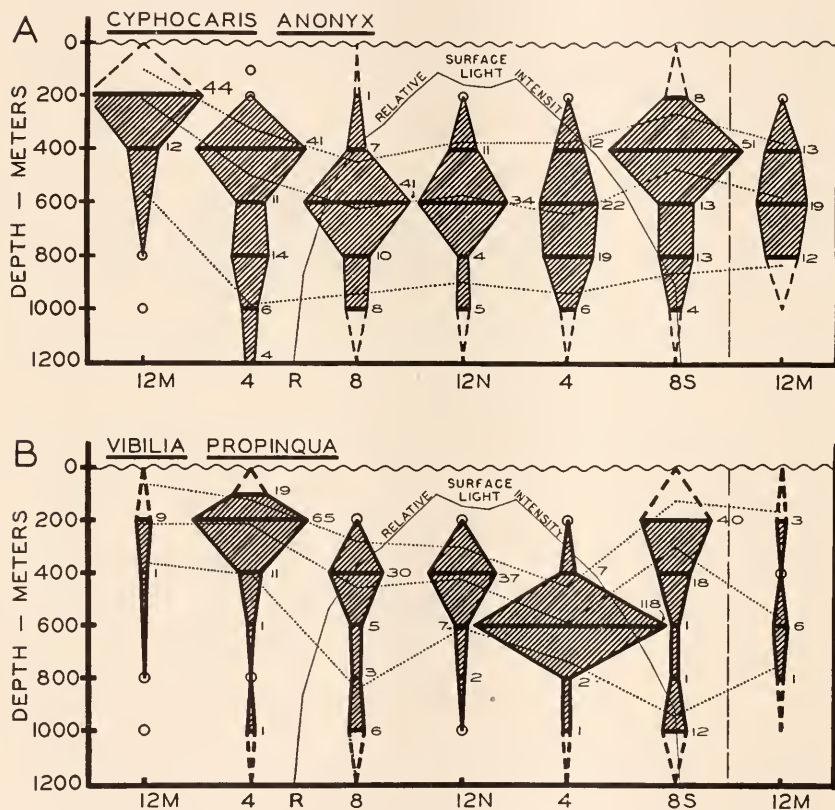


Fig. 5. Amphipods. Adults. See Fig. 2 for further explanation.

The two amphipods caught in sufficient numbers, *Cyphocaris anonyx* Boeck (Fig. 5A) and *Vibilia propinqua* Stebbing (Fig. 5B), also show a diurnal vertical migration of about 400 m. amplitude. In the case of *Vibilia*, however, the considerable amount of swarming which is apparent obscures this migration somewhat.

DISCUSSION

Since the hauls were made in such a way that our data give us information about points separated in effect by 200 m. and 4 hours, a detailed analysis of the diurnal vertical migrations is clearly impossible. Nevertheless, our results show that all of the crustaceans investigated were accomplishing vertical migrations 200 to 400, and possibly 600 m. in extent, which is in general agreement with the results of recent investigators.

For instance, certain of the larval stages of euphausiids taken by Fraser (1936, p. 154) were apparently moving upward 200–250 m. to reach the surface at night. Stubbings (1938, p. 27) found that species of the pteropods, *Creseis* and *Hyalocylis*, underwent a diurnal migration of 200 m. vertical extent and that some of the other pteropods may have been migrating even further. The copepod, *Pleuromamma robusta*, was shown by Mackintosh (1934, p. 92) to exhibit the most pronounced vertical migration of all the plankton animals of the antarctic surface waters; this crustacean rose into the upper 100 m. around midnight and descended apparently below 600 m. during the day. Among the malacostracan crustaceans Hardy and Gunther (1935, p. 240) observed that *Euphausia frigida* and *E. triacantha* underwent a diurnal vertical migration of at least 200 m. and that the amphipod, *Parathemisto gaudichaudi*, migrated a vertical distance of about 60 m. in the course of its diurnal movement. Since the latter animal was taken within the upper 100 m. at all hours, this observation is similar to those made on other shallow water plankton. Clarke (1934a, Figs. 2 and 3), for example, showed that *Calanus finmarchicus* adults and copepodid stages IV and V exhibited a diurnal migration of about 100 m.

The rate of the diurnal vertical migrations may be roughly calculated from our results. If the speeds of downward movement of the centers of the populations for the various species, as shown in the figures, are taken to represent the average rates of movement for the organisms concerned, these are found to vary from 29 m. per hour for *Nematoscelis* to 125 m. per hour for *Thysanopoda*. The average for the eleven species of crustaceans is 67 m. per hour. The only comparable data are those of Hardy and Gunther (1935, p. 240) mentioned above for the migration of the two species of *Euphausia* in which the upward migration apparently occurred at a rate of 100–200 m. per hour.

In some of these organisms a slower upward movement during the day preceded the more rapid upward migration shortly before midnight. This phenomenon has been observed in shallow-water zoöplankton by a number of workers and has been explained on the basis of a gradual

light adaptation of the animals (see Russell, 1931, p. 402; Kikuchi, 1938, p. 37). In spite of this slow upward trend, however, our observations suggest that the upward migration in the early evening hours was as rapid as the downward movement in the early morning.

A comparison of the swimming speeds of the animals in the plankton with the rates of these diurnal vertical migrations would be of great interest. Unfortunately, there are no data available for these or comparable plankton organisms. Measurements have been made, however, of the rates of locomotion of several copepods and crustacean larvae (Russell, 1927, p. 221; Welsh, 1933). The latter found that *Centropages* would swim towards a light of an intensity that might be found 20-30 m. below the surface on a clear day at a rate of 82 m. per hour (actually measured over a path only 10 cm. long). As Parker (1902), Gardiner (1933), and Seiwel and Seiwel (1938) have shown, gravity alone causes copepods to sink at a rate of about 24 m. per hour.

Thus, if any organism to which the above quoted rate of sinking is applicable were migrating downward 400 m. in 8 hours, it would sink 192 m. merely through the effect of gravity and would have to swim downward through the remaining 208 m. at an average rate of 26 m. per hour. For the upward trip the gravitational effect would add the 192 m. to the distance to be swum (see Clarke, 1934*b*) so that the animal would have, in effect, to swim upward at a rate of 592 m. in 8 hours or 74 m. per hour. To consider it in another way, the organism in order to accomplish the downward migration would have to be actively swimming towards the bottom at the experimentally determined rate of 82 m. per hour for only about one-third of the 8-hour period, whereas in order to move upward to the original level in the same length of time it would have to be swimming towards the surface at this rate during 7 of the 8 hours.

As the pelagic malacostracans such as those discussed in this paper are known to be very active swimmers, they could presumably accomplish such vertical migrations with greater ease than the animal postulated above on the basis of data obtained from copepods. The Crustacea in our analysis are larger, moreover, and, other things being equal, would sink more rapidly through the water than the copepods so that an even greater proportion of their downward movement may be effected by the mere absence of any persistently oriented swimming activity. Many experiments have shown that several environmental variables, such as light and temperature, will affect the locomotor activity of zoöplankton, but, as Clarke (1934*b*) pointed out, these factors under natural conditions would seem to work in the wrong direction to explain distribution as it is found.

Since the great majority of previous workers have recognized that light is the most significant single environmental factor in diurnal vertical migrations, it is interesting to correlate these phenomena with the changes in the intensity of illumination at various hours of the day (Fig. 1). During the first hour after daylight became measurable, its intensity increased at an exceedingly rapid rate. Similarly, the illumination diminished at an equally rapid rate during the hour preceding the last measurement of the day. During the four hours in the middle of the day the illumination remained relatively constant and such changes in intensity as resulted from different degrees of cloudiness were of a much lower magnitude than those of early morning and late afternoon.

The most striking point which appears in a comparison of the biological and the light intensity data is that much of the migration apparently occurred when there was no light more intense than starlight even at the surface. Before sunrise most of the animals had already descended about half of their total downward distance, and several, such as *Boreomysis*, *Eucopia*, and *Parapasiphaë*, had reached the deepest point in their migration by sunrise. In most cases, furthermore, the greater part of the upward migration must have been accomplished between the time of sunset and midnight.

It is clear from these facts that the vertical migrations of these organisms cannot be explained simply on the basis of a negative phototropism which overshadowed a negative geotropism in the daytime and left it with a free hand at night.

Similarly the hypothesis that the animals aggregate by means of a tropistic mechanism (Michael, 1911; Russell, 1934) in a region of optimum light intensity and follow the latter as it moves vertically at various hours of the day apparently will not explain the situation here. Such an hypothesis is further weakened by the lack of evidence in the present case for a general vertical scattering of the population throughout the water column at night when it would be free of the concentrating effect of light.

Whether we assume that external environmental or internal physiological factors are controlling the diurnal migrations, the intensity of illumination penetrating from the surface must play an important rôle in these phenomena since it is the only daily environmental variable in deep water. It may act in combination with other external factors, such as temperature and gravity, since many investigators have shown experimentally that interactions of such factors may cause a tropistic reversal (Kikuchi, 1938, pp. 35-6). Or it may merely keep in phase cyclic internal physiological conditions of the organisms which themselves regulate these migrations (see Russell, 1927, p. 225; Welsh,

Chace, and Nunnemacher, 1937, p. 195). Many cases are known (Welsh, 1938, p. 136) of the persistence of internal diurnal rhythms under constant external conditions, but one would expect that in nature at least an occasional action of the external factor to which it owes its origin, in this case light, would be necessary to maintain the synchrony indefinitely.

A second problem is posed by the fact that most of the Crustacea here considered were about 200 m. lower in the water on the midnight of July 22 than they were at the same hour two or three days before. Furthermore, the maximum illumination at the surface on July 22 was 66 per cent greater than the maximum on July 19. Previous studies have shown that certain organisms appear to live at depths where the light intensity is of a particular magnitude, i.e., an optimum intensity for the organism in question; these organisms may, as a consequence, appear in shallower waters on dull days or in high latitudes (Russell, 1933, p. 569; Murray and Hjort, 1912, p. 664-6); thus the light intensity itself may have been responsible for the observed difference in distribution.

It is clear, however, that the amount of illumination could not explain so great a change in distribution. Since actual measurements down to 84 m. (Fig. 6) showed that the light is reduced by a factor of 10, for every 25 m. of water, the observed change in light intensity at the surface would result in a difference of only about 5.5 m. in the depth at which any given intensity of light would be found. Even in the clearest sea water yet measured (see below) this change in light intensity at the surface would result in a maximum change of only 50 m. in the depth to which any particular intensity of light would penetrate.

An alternative causal or contributory factor to the observed lower position of the plankton on July 22 may have been the storm of the previous day, but it is not likely that a relatively mild disturbance of this sort at the surface would be appreciable at a depth of over 200 m. Furthermore, Hardy and Gunther (1935, p. 268) conclude from their observations that, "... the state of the sea has little or no bearing on the number of organisms in the surface layers. . . ."

A more important point is the fact that simultaneous measurements on the horizontal patchiness of the plankton were not made. Consequently we cannot tell whether or not the observed difference might rather be due to the fact that on the later day we were sampling a different population with a different vertical distribution pattern from that of the previous days (Russell, 1933, p. 569; Hardy, 1936).

One of the most important questions raised by previous work on the diurnal vertical migrations of bathypelagic organisms is how deep

down in the water do such migrations occur. Most of the crustaceans showing diurnal vertical migrations discussed in this paper were living at depths of 600–800 m. during the four hours during the middle of the day. If, as pointed out above, we recognize the necessity of light in one way or another for the vertical migrations of these animals, we must assume that at some time during the day the organism is affected

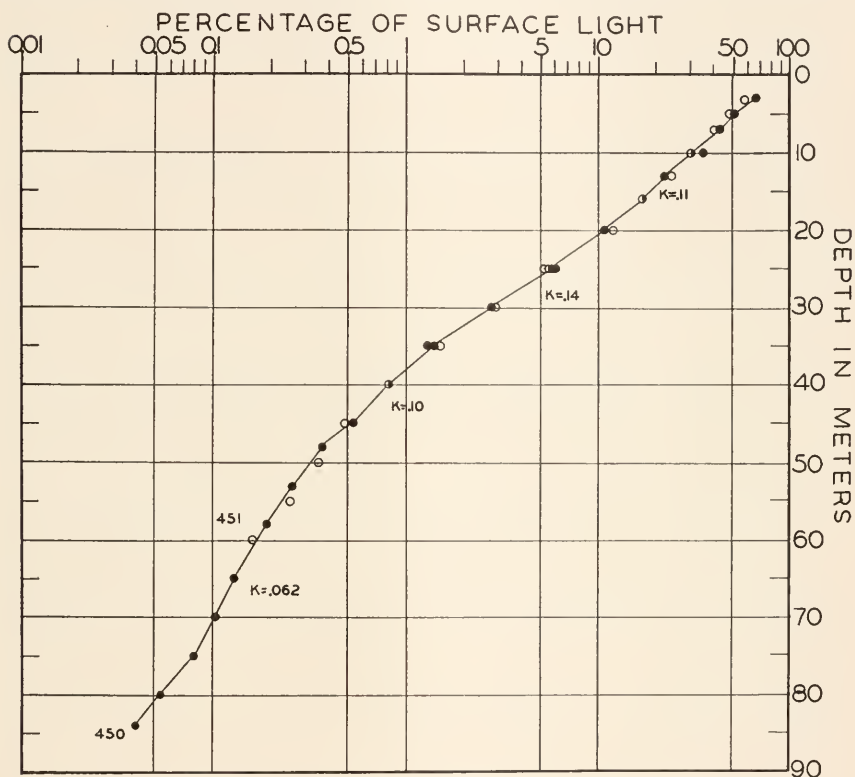


FIG. 6. Relation between depth and irradiation expressed as a percentage of the light just over the surface (logarithmic scale). Station 2894. Lat., $39^{\circ} 06' N.$; Long., $70^{\circ} 16' W.$ July 22, 1937. Sky: clear. Sea: moderate, white caps. Series 450 measured by photronic cell with opal disc. Series 451 measured by photronic cell with "green" filter combination (Schott-Jena BG-18 and GG-11) and opal disc.

by light. Clearly, under any given physiological conditions, there is a minimum intensity of illumination which would be just sufficient to affect any particular animal; in the open sea there would be a definite maximum depth beyond which this minimum light intensity would never penetrate even at noon on the brightest days. A diurnal vertical migration in which illumination from the surface was supposed to have any

controlling effect would thus be impossible below this depth. As pointed out above, however, vertical migrations could be temporarily maintained under constant conditions by a diurnal physiological rhythm within the organism. Thus, if unusual conditions such as a storm should cut down the incident light so that deep-living animals did not come under the influence of light for several days, there would not be a wholesale loss of the population through continued downward migration or random scattering into unusually great depths (see Russell, 1930), but the diurnal migrations would continue more or less as usual under the guidance of the physiological rhythm.

In view of these facts the question of how much light penetrates to the depths at which the migrating Crustacea are living during the daylight hours is one of great interest. The results of our two series of light penetration measurements, shown in Fig. 6, indicate that the transparency of the water was relatively uniform down to 84 m., the limit of measurement, with an average value of the extinction coefficient ⁶ of $k = 0.092$ for the green component of daylight. Hence, the intensity of illumination was reduced to one-tenth its value by passage through 25 m. of water. Assuming that the transparency of the water remained the same below the depth of our deepest measurement and extrapolating we find that the illumination at 800 m. would be 10^{-32} times its intensity at the surface.

But if we assume that the water below 100 m. is as clear as any sea water which has been measured, we may on this basis calculate the maximum amount of light possible at any depth. Since the extinction coefficient varies enormously with wave-length, the actual result reached will depend on the part of the spectrum considered. In the clearest water yet known the intensity of light in the green wave-lengths (Clarke, 1938*b*) was reduced to one-tenth by 61 m. of water. Thus if the water below 100 m. at our station was as clear as this, at 800 m. the intensity of illumination would be 10^{-15} times the value at the surface. In the clearest water yet known the blue wave-lengths were reduced to one-tenth their intensity in 230 m. (Clarke, 1933*a*).⁷ If water of like transparency existed below 100 m. at our station, the illumination of 800 m. would be a little less than 10^{-7} times the surface intensity;⁸ this value is between 100 and 1,000 times higher than the threshold intensity for human vision.

⁶ In the equation $I/I_0 = e^{-kL}$, where L is the thickness of the water layer in meters through which the intensity is reduced from I_0 to I .

⁷ Basing our calculations on the most transparent strata only.

⁸ Assuming the same reductions in the upper 100 m. for the blue component as for the green, which is known (Oster and Clarke, 1935) to be approximately true in this region of the Atlantic.

We have some evidence, however, that conditions as favorable for penetration as this latter case for blue light did not exist at our slope water station since on a bright, calm day Beebe (1934, p. 276) observed through the window of the bathysphere that at a depth of 610 m., the water appeared absolutely dark to the human eye. But Beebe was not able to look upward in the water so that he could not tell at what depth the downward component of the light had become indistinguishable from the blackness below.⁹ On the other hand, the water of the Sargasso Sea, where Beebe made his observations, is characteristically (Clarke, 1936a) nearly twice as transparent in the upper 100 m. for the green region of the spectrum as was the water at our present station.

It would be of great interest to compare the amounts of light present at 800 m., where migrations were definitely going on, with the visual sensitivity of these Crustacea. Unfortunately, no experimental studies have yet been made on vision in any deep-sea animals. Nevertheless, from previous work on crustaceans and other arthropods with compound eyes, it is possible to draw two extremely general conclusions: first, that arthropods are more sensitive to the blue wave-lengths of light than to the green (see Waterman, 1937, p. 464 for table summarizing pertinent data) and second, that for these organisms the minimal light intensity for response is of the order of magnitude of 10^{-6} to 10^{-7} millilamberts (Crozier, 1938, personal communication).¹⁰

An intensity of illumination of 10^{-6} millilamberts is equivalent to about 10^{-10} times that of full noon sunlight and is the value used by Clarke (1936b) as a threshold intensity for determining the depth at which fish can see. A crustacean with a visual threshold of such a magnitude in the green wave-lengths certainly could not be stimulated by this region of the spectrum when the intensity of the latter is reduced to 10^{-15} times that at the surface. Since this would be the illumination present at 800 m., when we assume that the water below 100 m. was as clear as any sea water that has been observed, we may conclude that under these conditions the crustaceans could not be re-

⁹ Presumably this directional effect would still be appreciable at great depths since directional differences in intensity were recorded photographically by Helland-Hansen (Murray and Hjort, 1912, p. 252) at 500 m. and since more recent work of Whitney (1938, p. 211) has demonstrated that in optically homogeneous waters the ratio of the intensities of light passing downward to those of the light scattered upward is a constant (see also Pettersson, 1938). Of interest in this connection is the occurrence of upwardly (dorsally) directed "telescopic" and stalked eyes in a number of different families of deep-sea fishes of the "twilight zone" of the seas (Brauer, 1908, p. 233) which would suggest that this directional difference in the light intensities is of biological significance even at considerable depths.

¹⁰ Any value obtained for a sensory threshold will, of course, depend largely on the exact experimental conditions under which it was determined.

sponding to light from the surface. But if the crustacean had an equally low visual threshold for blue light, our calculations have shown that in the clearest water observed there would be almost 1,000 times as much light present at 800 m. as would be necessary for the threshold of vision. Under such maximum conditions even at 1,000 m., where the immature *Hymenodora* in our catches possibly gave some indications of a diurnal vertical migration, more than 100 times the necessary amount of light would be present. The threshold intensity for vision, 10^{-10} of full sunlight intensity, would occur at a depth of nearly 1,500 m. under such conditions.

Thus, the water below the surface layers could be, as it probably was, considerably less transparent than the clearest sea water ever observed and still permit sufficient visible light for affecting the reactions of bathypelagic organisms to penetrate to depths of about 800 m.

The preceding discussion makes it clear that several important aspects of this problem must be investigated more thoroughly before a satisfactory solution will be possible. These are in brief: (1) actual measurements of the transparency of the water at greater depths and of the relative intensities of the light in various directions; (2) more detailed work on the maximum depth at which diurnal vertical migrations occur; (3) measurements of the photosensitivity of deep-sea animals and correlations of the maximum wave-length sensitivity to the wave-length of the most penetrating part of the spectrum; (4) experimental studies on deep-sea animals designed to elucidate the nature of any diurnal physiological rhythms and their relation to the organisms' behavior in the sea.

SUMMARY

1. A study was made with the aid of closing nets of the diurnal vertical migrations of bathypelagic organisms at a station in continental slope water of the western North Atlantic.

2. While the hauls were being made a continuous record of the light intensity at the surface was kept.

3. The penetration of light into the upper 84 m. of water was directly measured photometrically; the average extinction coefficient for green light was $k = 0.092$.

4. All of the malacostracan Crustacea (to which the detailed results presented in this paper are limited) which occurred in sufficient numbers for analysis exhibited diurnal migrations 200 to possibly 600 m. in vertical extent.

5. The speed of vertical movement in these migrations varied from 24 to 125 m. per hour among the various crustaceans.

6. A considerable part of the migrations took place while the light intensity even at the surface was no greater than starlight.

7. Several Crustacea living at 800 m. during the day showed extensive diurnal vertical migrations.

8. It is concluded, however, that whether the migrations are regulated by external environmental or by internal physiological factors, at some time of day the organisms concerned are affected by light penetrating from the surface.

9. Calculations made from the light penetration data indicate that the amount of light probably present during the middle of the day at the depths where the animals were migrating was adequate to support this conclusion (8).

LITERATURE CITED

- BEEBE, W., 1934. Half Mile Down. Harcourt, Brace. New York.
- BRAUER, A., 1906. Die Tiefsee-Fische. I. Systematischer Teil. *Wiss. Erg. Deutschen Tiefsee-Exp.* . . . "Valdivia," Bd. 15, Lief. 1.
- BRAUER, A., 1908. Die Tiefsee-Fische. II. Anatomischer Teil. *Ibid.*, Bd. 15, Lief. 2.
- CHIERCHIA, G., 1885. Collezioni per studi di scienze naturali fatti . . . dalla . . . "Vettor Pisani." *Riv. Marittima*, Sept. 1885.
- CHUN, C., 1890. Die pelagische Thierwelt in grossen Meerestiefen. *Verhandl. Gesell. Deut. Naturf. u. Aerzte*, 63 (Pt. 1): 69-85.
- CLARKE, G. L., 1933a. Observations on the penetration of daylight into mid-Atlantic and coastal waters. *Biol. Bull.*, 65: 317-37.
- CLARKE, G. L., 1933b. Diurnal migration of plankton in the Gulf of Maine and its correlation with changes in submarine irradiation. *Ibid.*, 65: 402-436.
- CLARKE, G. L., 1934a. Further observations on the diurnal migration of copepods in the Gulf of Maine. *Ibid.*, 67: 432-55.
- CLARKE, G. L., 1934b. Factors affecting the vertical distribution of copepods. *Ecol. Monogr.*, 4: 530-40.
- CLARKE, G. L., 1936a. Light penetration in the western North Atlantic and its application to biological problems. *Rapp. et Procès-verb. Con. Perm. Int. Expl. Mer*, 101 (Pt. 2, No. 3): 3-14.
- CLARKE, G. L., 1936b. On the depth at which fish can see. *Ecol.*, 17: 452-6.
- CLARKE, G. L., 1938a. Seasonal changes in the intensity of submarine illumination off Woods Hole. *Ibid.*, 19: 89-106.
- CLARKE, G. L., 1938b. Light penetration in the Caribbean Sea and in the Gulf of Mexico. *Jour. Mar. Res.*, 1: 85-94.
- EKMAN, S., 1935. Tiergeographie des Meeres. Akademische Verlagsgesellschaft. Leipzig.
- FOWLER, G. H., 1905. Appendix on the vertical distribution and movement of the Schizopoda. *Trans. Linn. Soc. Lond.*, Ser. 2, 10: 122-9.
- FOWLER, G. H., 1909. Biscayan plankton collected during a cruise of H.M.S. "Research," 1900. Pt. 12: The Ostracoda. *Ibid.*: 219-336.
- FRASER, F. C., 1936. On the development and distribution of the young stages of krill (*Euphausia superba*). *Discov. Reports*, 14: 1-192.
- GARDINER, A. C., 1933. Vertical distribution in *Calanus finmarchicus*. *Jour. Mar. Biol. Ass'n, N.S.*, 18: 575-610.
- HARDY, A. C., 1936. Observations on the uneven distribution of oceanic plankton. *Discov. Reports*, 11: 511-38.

- HARDY, A. C., AND E. R. GUNTHER, 1935. The plankton of the South Georgia whaling grounds and adjacent waters, 1926-1927. *Ibid.*, **11**: 1-456.
- ISELIN, C. O'D., 1936. A study of the circulation of the western North Atlantic. *Papers in phys. oceanogr. and meteorol. Mass. Inst. Tech., Woods Hole Oceanogr. Inst.*, **4** (No. 4): 1-101.
- KIKUCHI, K., 1938. Studies on the vertical distribution of the plankton Crustacea. II. The reversal of phototropic and geotropic signs of the plankton Crustacea with reference to vertical movement. *Rec. Oceanogr. Works in Japan*, **10**: 17-41.
- LEAVITT, B. B., 1935. A quantitative study of the vertical distribution of the larger zoöplankton in deep water. *Biol. Bull.*, **68**: 115-30.
- LEAVITT, B. B., 1938. The quantitative vertical distribution of macrozoöplankton in the Atlantic Ocean basin. *Ibid.*, **74**: 376-394.
- MACKINTOSH, N. A., 1934. Distribution of the macroplankton in the Atlantic sector of the Antarctic. *Discov. Reports*, **9**: 65-160.
- MICHAEL, E. L., 1911. Classification and vertical distribution of the Chaetognatha of the San Diego region. *Univ. Calif. Publ. Zool.*, **8**: 21-186.
- MURRAY, J., 1885. In *Reports of the "Challenger" Exped.* Vol. 1, Narrative of the Voyage, p. 218.
- MURRAY, J., AND J. HJORT, 1912. *The Depths of the Ocean*. Macmillan Co. London.
- OSTER, R. H., AND G. L. CLARKE, 1935. The penetration of the red, green, and violet components of daylight into Atlantic waters. *Jour. Opt. Soc. Amer.*, **25**: 84-91.
- PARKER, G. H., 1902. The reactions of copepods to various stimuli, and the bearing of this on daily depth migrations. *Bull. U. S. Fish. Comm.* 1901, **21**: 103-23.
- PETERSSON, H., 1938. Measurements of the angular distribution of submarine light. *Rapp. et Procès-verb. Con. Perm. Int. Expl. Mer*, **108** (Pt. 2, No. 2): 9-12.
- POOLE, H. H., 1938. Review of W. M. Powell and G. L. Clarke, 1936. *Jour. Con. Perm. Int. Expl. Mer*, **13**: 111-3.
- RANG, M., 1828. Notice sur quelques Mollusques nouveaux appartenant au genre Cléodore, et établissement et monographie du sous-genre Créseis. *Ann. des Sci. Nat.*, **13**: 302-19.
- ROSE, M., 1925. Contribution à l'étude de la biologie du plankton; le problème des migrations verticales journalières. *Arch. Zool. Expér. Gén.*, **64**: 387-542.
- RUSSELL, F. S., 1927. The vertical distribution of plankton in the sea. *Biol. Rev.*, **2**: 213-62.
- RUSSELL, F. S., 1930. Do oceanic plankton animals lose themselves? *Nature*, **125**: 17.
- RUSSELL, F. S., 1931. The vertical distribution of marine macroplankton. X. Notes on the behaviour of *Sagitta* in the Plymouth area. *Jour. Mar. Biol. Ass'n, N.S.*, **17**: 391-414.
- RUSSELL, F. S., 1933. On the biology of *Sagitta*. IV. Observations on the natural history of *Sagitta elegans* Verrill and *Sagitta setosa* J. Müller in the Plymouth area. *Ibid.*, **18**: 559-74.
- RUSSELL, F. S., 1934. The vertical distribution of marine macroplankton. *Ibid.*, **19**: 569-84.
- SEIWELL, H. R., AND G. E. SEIWELL, 1938. The sinking of decomposing plankton in sea water and its relationship to oxygen consumption and phosphorus liberation. *Proc. Amer. Phil. Soc.*, **78**: 465-81.
- SIGSBEE, C. D., 1880. Description of a gravitating trap for obtaining specimens of animal life from intermediate ocean-depths. *Bull. Mus. Comp. Zool.*, **6**: 155-8.

- STUBBINGS, H. G., 1938. Pteropoda. *John Murray Exp. Sci. Rep.*, 5: 15-33.
- WATERMAN, T. H., 1937. The relative effectiveness of various wave lengths for the photokinesis of *Unionicola*. *Jour. Cell. and Comp. Physiol.*, 9: 453-67.
- WELSH, J. H., 1933. Light intensity and the extent of activity of locomotor muscles as opposed to cilia. *Biol. Bull.*, 65: 168-74.
- WELSH, J. H., 1938. Diurnal rhythms. *Quart. Rev. Biol.*, 13: 123-39.
- WELSH, J. H., AND F. A. CHACE, JR., 1937. Eyes of deep sea crustaceans. I. Acanthephyridae. *Biol. Bull.*, 72: 57-74.
- WELSH, J. H., AND F. A. CHACE, JR., 1938. Eyes of deep-sea crustaceans. II. Sergestidae. *Ibid.*, 74: 364-75.
- WELSH, J. H., F. A. CHACE, JR., AND R. F. NUNNEMACHER, 1937. The diurnal migration of deep-water animals. *Ibid.*, 73: 185-96.
- WHITNEY, L. V., 1938. Transmission of solar energy and the scattering produced by the suspensoids in lake waters. *Trans. Wisconsin Acad.*, 31: 201-21.

THE REPRODUCTION OF LIMACINA RETROVERSA (FLEM.)

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INTRODUCTION

The structure of the reproductive system of *Limacina retroversa* and the process of spermatogenesis of this species have been described in a previous paper (Hsiao, 1939). It was shown that very small animals are sexually undifferentiated. The gonads of sexually differentiated but small-sized individuals are protandric and many of them are often without female tissue, that is, "pure male," while those of the bigger animals are essentially hermaphroditic, showing different proportions of male and female tissues. Some individuals function as male, others as female and still others both as male and female at the same time. It is the purpose of this study to examine the relationship between size and sexual phases and the time of the occurrence of the latter so as to determine their sequence in the reproductive history of this form and to compare it with other mollusks whose sexual history is known.

DESCRIPTION OF MATERIAL

The material was collected by the research vessel "Atlantis" during a series of cruises in the Gulf of Maine between June, 1933 and September, 1934. Specimens collected at the following periods were used:

Date	Designated as
December 2-11, 1933	December specimens
January 9-13, 1934	January specimens
March 21-28, 1934	March specimens
April 17-May 13, 1934	April specimens
May 21-June 2, 1934	May specimens
June 25-July 1, 1934	June specimens
September 17-24, 1934	September specimens

The specimens were caught by vertical hauls made with a 1.5-meter Heligoland larva net drawn from a level near the bottom up to the surface and fixed on board the ship in 2 per cent formalin in sea water.

¹ Contribution No. 206 from the Woods Hole Oceanographic Institution.

Limacina retroversa were later sorted out from the other species and specimens were selected from one to three stations from each cruise so as to make a series covering nearly the whole range of size. Each individual was measured with shell-opening facing the observer under a binocular microscope with a calibrated ocular micrometer. The diameter of the largest whorl of the spiral body of the animal, excluding the protruding margin of the shell-opening ($A-B$ in Fig. 1), was measured and used to designate the size of the specimens studied.

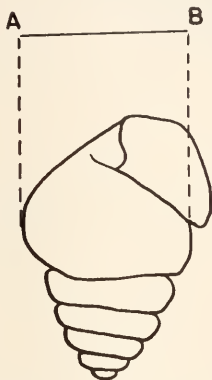


FIG. 1. Camera lucida drawing of the shell of *Limacina retroversa*. $\times 20$. $A-B$: maximum diameter of the shell used for comparison of size.

From a study of the size distribution of *Limacina retroversa* from 1933-34 and the movement of the water masses, Redfield (1939) has shown that at least two different populations entered the Gulf of Maine during the period of observation. The first group, called "population A," invaded the Gulf during December, 1933 and January, 1934, and later in the spring a second group, "population B," entered the basin during April and May from the same general direction. The positions of the hydrographic stations from which specimens used in this study were taken are shown in Fig. 2. In this map the solid circles with the abbreviations of the months beside them show the position and time of collection of the material from the population of *Limacina retroversa*, which has been identified as "population A" by Redfield. The positions of the stations providing the material fall within the region where large numbers of "population A" were found (as shown by Redfield's map), that is, in or near the center of density of this population in the different months. On the same map (Fig. 2) the open circles, with the abbreviations of the months in parentheses under them, indicate the stations in April and May from which specimens were selected which repre-

sent "population B." From these specimens the reproductive history of the species is worked out. The following table (Table I) shows the number of individuals used from each cruise grouped into size classes with a class interval of 0.3 mm.

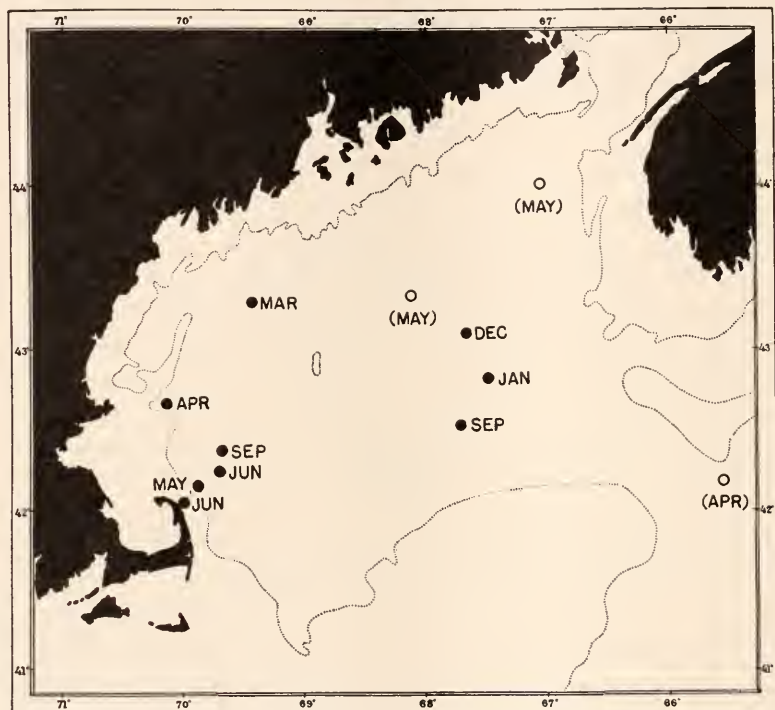


FIG. 2. Map of the Gulf of Maine showing the hydrographic stations from which samples of *Limacina retroversa* are selected for microscopic study.

- : stations for "population A."
- : stations for "population B."

The method employed in making serial sections of these specimens has been described in a previous paper. The following observations are based upon a comparative study of these serial sections.

RELATION BETWEEN SIZE AND SEX

The relation between size and sex in *Limacina retroversa* has been examined from two different angles: (1) The gonadal types and size, and (2) the relation between functional types and size.

Gonadal Types and Size

The following morphological types are seen among *Limacina*. (a) Sexually undifferentiated individuals all of which are small-sized; (b) "pure males" which are nearly completely male in constitution and are somewhat larger; and (c) hermaphrodites, which have various proportions of male and female tissues in the ovotestis and are generally of large size. The occurrence of the different morphological types of individuals in *Limacina retroversa*, in order of size, is shown in Table II.

Sexually Undifferentiated Individuals.—Of all the *Limacina retroversa* sectioned the largest individual which has a gonad still in a sexually undifferentiated condition measures 0.85 mm. in diameter. Indi-

TABLE I

Table showing the number of individuals from each period of collection which were used in this study. The individuals are separated according to size into groups with a class interval of 0.3 mm.

Month of cruise	Size groups in mm.								Total number obtained during month
	0 to 0.3 mm.	0.3 to 0.6 mm.	0.6 to 0.9 mm.	0.9 to 1.2 mm.	1.2 to 1.5 mm.	1.5 to 1.8 mm.	1.8 to 2.1 mm.	2.1 to 2.5 mm.	
Dec.....	3	13	9	8					33
Jan.....		13	12	4	3				32
Mar.....		4	4	7	10	1			26
Apr.....			5	9	5	6	1		26
May.....	4	11	7	6	3	6	2	1	40
June.....	4	3	6	2	6	5	2		28
Sept.....	1	6	8	7	4	3			29

viduals larger than this size all show definite gametogenesis. All specimens with a diameter less than 0.6 mm. are sexually undifferentiated. Hence, the process of sexual differentiation must commence during the period when the animal is between 0.6 mm. and 0.85 mm. in diameter. This is brought out more clearly when the percentage of sexually undifferentiated individuals is plotted against size as shown in Fig. 3.

"Pure Males."—These are animals with gonads which contain little or no noticeable amount of ovarian tissue. They correspond to Coe's "true males" or Orton's "pure males." These individuals are confined to comparatively small-sized groups. Table II, column 8, shows the sudden appearance and the steady but more gradual decrease of "pure males" among animals arranged in the order of increasing size. Among larger animals this type of individual gives place to those which have

various proportions of feminine reproductive cells in the gonads. These are the hermaphrodites described in the next paragraph. On the average, about 22 per cent of the specimens less than 1.2 mm. in diameter belong to the "pure male" class. Many of these males are functional at the time of fixation as shown by the presence of mature spermatozoa in the gonoduct.

Animals with Various Proportions of Male and Female Tissues in the Gonads.—In addition to the sexually undifferentiated animals and the "pure males" a small number of animals less than 1 mm. in diameter

TABLE II

Proportion of different morphological types of *Limacina retroversa* separated into groups without regard to the time of collection. Animals smaller than 1.2 mm. in diameter are separated into 0.1 mm. classes, those larger than 1.2 mm., into 0.3 mm. classes.

1	2	3	4	5	6	7	8	9	10
Size in mm.	Total number	Undifferentiated	Pure male	>50% male	<50% male	Percentage undifferentiated	Percentage pure male	Percentage of >50% male	Percentage of <50% male
0.4	3	3	0	0	0	100	0	0	0
0.5	3	3	0	0	0	100	0	0	0
0.6	13	8	3	2	0	62	23	16	0
0.7	15	5	7	3	0	33	47	20	0
0.8	24	3	8	13	0	13	33	54	0
0.9	12	0	3	7	2	0	25	58	17
1.0	14	0	2	8	4	0	14	57	28
1.1	15	0	2	10	3	0	13	67	20
1.2 to 1.49	44	0	0	34	10	0	0	77	23
1.5 to 1.79	34	0	0	26	8	0	0	77	23
1.8 to 2.09	19	0	0	11	8	0	0	58	42
2.1 to 2.39	4	0	0	2	2	0	0	50	50
2.4 to 2.7	2	0	0	1	1	0	0	50	50

may have gonads containing various proportions of male and female tissues at the same time. Of all the animals greater than 1.1 mm. in diameter no undifferentiated or "pure males" were found. They all contain spermary and ovarian tissues in the same gonad. Following the criteria described in a previous paper these hermaphroditic animals can be classified as hermaphroditic males or hermaphroditic females according to whether they have more than 50 per cent or less than 50 per cent of their gonadal tissues consisting of male reproductive elements. The proportion of these two kinds of individuals in the different size groups is

shown in Table II. Column 5 shows the number of individuals with more than 50 per cent of their gonadal tissue made up of masculine elements, excluding those which are already listed in column 4 as "pure males." It shows the number of hermaphroditic males only. The percentages of hermaphroditic males of this class are given in column 9. Column 10 shows the percentages of hermaphroditic females based on the numbers shown in column 6. A comparison of columns 9 and 10 will show that the minimal size of the hermaphroditic females is much greater than that of the hermaphroditic males. This occurrence of hermaphroditic males as well as "pure males" in smaller-sized animals when compared with the hermaphroditic females indicates that the male reproductive tissue is developed when the animals are smaller than is the case with the female cells of reproduction.

Owing to the small number of individuals in each size group among the larger animals the data in the lower half of Table II have been rearranged so that the class interval is three times as large as in the upper half. It will be seen that among animals smaller than 1.2 mm. the proportion of hermaphroditic males increases steadily while among those larger than this size the percentages decrease from 77 to 50. On the other hand, there is only a small proportion of hermaphroditic females among the smaller animals, but among the larger ones the percentage increases from 23 to 50. Approximately speaking, before the animals reach 1 mm. in diameter the proportion of males increases very rapidly, indicating an early proliferation of the male reproductive cells giving rise to the types of males whose gonads are predominantly masculine in appearance. After the animals grow to a size greater than 1.5 mm. in diameter the female reproductive cells overtake the male in development, thus bringing about a decrease in the proportion of hermaphroditic males after the 1.8 mm. size group and an increase in the predominantly feminine type. The decrease in the proportion of animals which are predominantly masculine continues until the animals are about 2 mm. in diameter, from which stage onward the two sexual types are equally numerous (Fig. 3).

The Relation between Functional Types and Size

In addition to the above morphological types, we can analyse our material into functional types. From the structure and the place of occurrence in the body of mature germ cells each *Limacina's* state of reproductive activity at the time of fixation can be deduced. Thus, when mature spermatozoa are seen in the gonoduct, or at the base of the penis, the individual is obviously functioning as a male and can be classified as an *active male*. When mature ova are found in the ovotestis the indi-

vidual can be considered as a *mature female*, while the presence of ripe eggs in the gonoduct indicates that the individual is a *spawning female*. The simultaneous presence of mature spermatozoa in the gonoduct and ripe ova in or out of the gonad is an indication that the individual is a *functional hermaphrodite*, to use Coe's terminology. The occurrence of these functional types is shown in Table III.

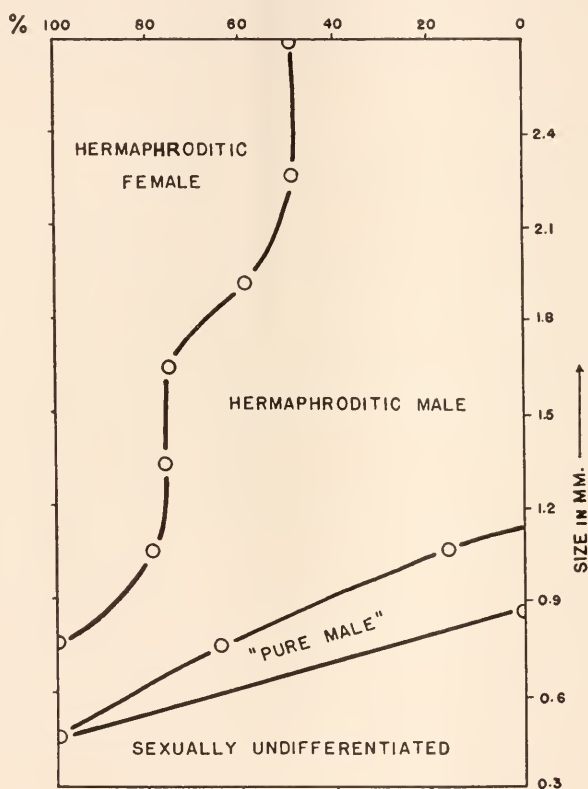


FIG. 3. Diagram showing the relation between size and various morphological types of gonads of *Limacina retroversa* (expressed in percentages of the total of each size group).

It has been observed that some individuals as small as 0.7 mm. in diameter may function as males. But the majority of specimens actively functioning as males only are between 1.2 mm. and 1.8 mm. in diameter (column 4). In larger-sized groups their place is taken by the females and individuals functioning both as male and female simultaneously.

The proportion of mature females with ripe eggs in the gonad, on the contrary, increases with the increase of size. The class with the

largest number of mature females, as shown in Table III, is the 2.1 to 2.4 mm. group. The smallest *Limacina* with mature ova is more than 1 mm. in diameter. At the time of fixation many mature females were in the act of spawning, as evidenced by the presence of ripe ova at various points along the gonoduct. Roughly speaking, about half of the mature females in each size group were in the act of spawning when fixed.

The proportion of the third functional type, functional hermaphrodites, in each size group is shown in column 6 of Table III. The chief occurrence of this type of animal is among the larger-sized specimens. It will be seen from Table III that although both specimens of the 2.4–2.7 mm. size group are functional hermaphrodites, the number of individuals used is so small that no great importance can be attached to the

TABLE III

Proportion of different functional types of *Limacina retroversa* expressed as percentage of each size group irrespective of the time of collection. Class interval: 0.3 mm.

1	2	3	4	5	6	7
Size groups (mm.)	Total number	Undiffer- entiated	Active male	Mature female	Functional hermaphro- dite	Without signs of activity
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.3–0.6	6	100	0	0	0	
0.6–0.9	52	30	17	0	0	53
0.9–1.2	41	0	39	15	5	41
1.2–1.5	44	0	48	9	14	29
1.5–1.8	43	0	47	18	21	14
1.8–2.1	19	0	5	42	10	43
2.1–2.4	4	0	0	50	50	0
2.4–2.7	2	0	0	0	100	0

deduced percentage of this type of animal. Until more large specimens are studied it seems better to leave this group out in our examination of the relation between the functional types and size. In the 2.1–2.4 mm. size group mature females and functional hermaphrodites are equally numerous.

When the gonads of these functional hermaphrodites are examined and classified in terms of the proportion of masculine and feminine tissue in them the following relationship is seen.

Type of gonad:	Percentage of specimens
With more than 75% male tissue	57%
With 50%–75% male tissue	33%
With 25%–50% male tissue	10%
With less than 25% male tissue	0%

Most functional hermaphrodites have more than 75 per cent of the gonadal tissue made of male reproductive cells, one-third of them have a 50 per cent to 75 per cent male type of gonad, while no *Limacina* with less than 25 per cent male tissue in the gonad, that is, no very pronounced female type of individual, functions as hermaphrodite. Since the gonad of this species of pteropod changes from predominantly male to predominantly female type as the animals grow (see Fig. 3), this relation between the proportion of functional hermaphrodites and the amount of masculine tissue in the gonad can be interpreted to mean that animals possessing "more than 75 per cent male" type of gonad later have their female germ cells developed while the male phase of reproduction is still functioning, thus giving rise to a large proportion of hermaphrodites functioning as male and female at the same time. By the time the gonads change over to the predominantly feminine condition the male phase of sexual activity also disappears and hence few or no functional hermaphrodites are seen among individuals with a pronounced feminine type of gonad.

Figure 3 summarizes the facts about the relation of size to sexuality. Sexual differentiation commences during the time when the animals are between 0.6 and 0.9 mm. in diameter. The male germ cells proliferate in smaller-sized individuals giving rise to the "pure males" and hermaphroditic males before the animal reaches 1.2 mm. in diameter. The proportion of "pure males" decreases with increase in size. As the size increases further, the proportion of hermaphroditic males decreases, while that of the hermaphroditic females becomes greater and greater until the two sexual types are equal in number among animals greater than 2 mm. in diameter.

Functionally the situation is summarized in Fig. 4. The male phase starts among smaller *Limacina* than the functional female and hermaphroditic phases. The highest proportion of actively functioning males is found among animals 1.2 to 1.8 mm. in diameter. The large proportion of mature females and functional hermaphrodites occurs among larger animals replacing the male phase in those size groups. Females become mature after they have attained a diameter greater than 1 mm. It is probable that mature females and functional hermaphrodites are equally numerous among animals greater than 2 mm. in diameter. The percentage of each of these three functional types among the different size groups is shown in Fig. 4.

SEASONAL CHANGES IN THE REPRODUCTIVE CYCLE

When the condition of the reproductive system of individuals collected at different times of the year is examined comparatively it is found

that, as a general rule, in winter individuals are small protandric males and that later hermaphrodites and functional males and females appear. We shall first take up the morphological types of sexual individuals and the question of the proportion of these sexual types in different months, then the appearance of different sexual activities at various times of the year and lastly the question of the reversal of sex predominance. It has

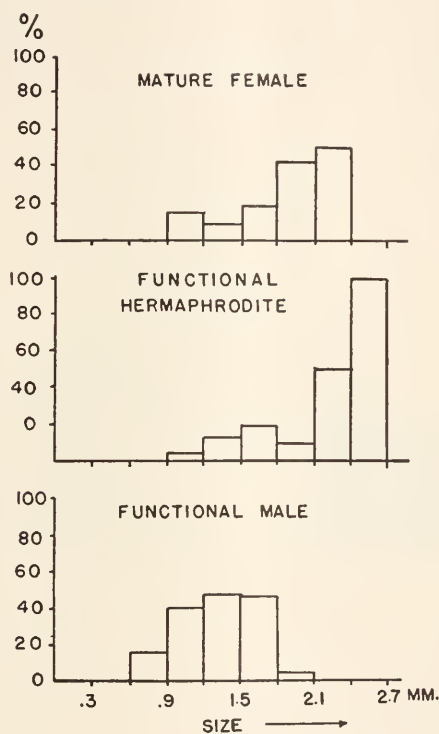


FIG. 4. Distribution of the three functional types of *Limacina retroversa* among various size groups. Functional males occur among smaller-sized animals than functional hermaphrodites and mature females.

been shown by Redfield (1939) that "population A" can be clearly differentiated from "population B" from December up to May and after that time there is a good deal of mixing of populations with "population B" predominating over the descendants and remnants of "population A" and new recruits into the Gulf. In the following description data from December to May refer to "population A" and those after May, to the mixed populations.

Change in Gonad Structure

Young sexually undifferentiated individuals were found among the specimens taken in each month except March and April. Figure 5 shows the percentage of the individuals taken each month which are sexually indefinite. During the winter months of December and January "population A," which entered the Gulf then, contained 14–16 per cent of sexually undifferentiated animals. In March and April these animals drifted to the western portion of the Gulf and grew larger, as shown by Redfield, and there remained no sexually undifferentiated individuals. After May, a portion of the animals examined, 19–20 per cent, were

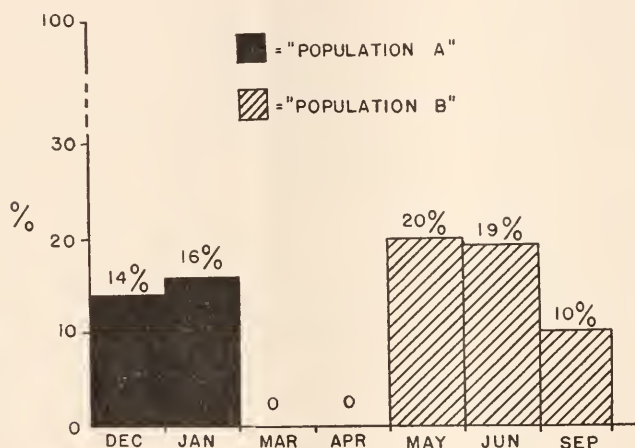


FIG. 5. Histogram showing the percentage of sexually undifferentiated individuals in each month. "Population A" solid black, "population B" shaded with straight lines. About 15 per cent of the material caught (in the winter) from "population A" and 20 per cent from "population B" are sexually undifferentiated. Absence of immature material in March and April indicates that members of "population A" are all differentiated by March.

again sexually indefinite. This coincides with the entrance into the Gulf of a new population in the east and the spawning of "population A" in the west portion of the basin. As far as "population A" is concerned, sexual differentiation is completed by the month of March and all the individuals of this population were either developing in the direction of males or hermaphrodites.

Animals which were sexually differentiated have been divided into male or female type according to whether they had more or less than 50 per cent of their gonadal tissue made of male reproductive cells. The proportions of these male and female types among sexually differentiated *Limacina* collected each month are shown in Fig. 6. In this figure the

material before May refers to "population A" and that after May to the mixed populations, with "population B" as the predominating constituent. It will be seen that in December and January nearly all the sexually differentiated *Limacina* were predominantly male. After these months the proportion of predominantly male individuals became reduced: in April they constituted only 40 per cent, while in May the two sexual types were equally numerous. After May the proportion of predominantly male animals became more numerous again among the mixed populations.

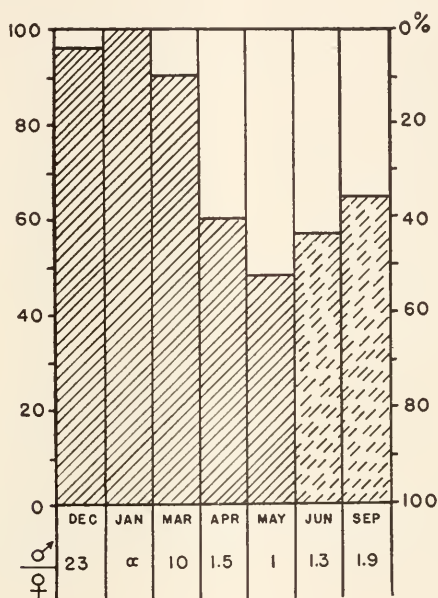


FIG. 6. Proportions of hermaphroditic males and hermaphroditic females among sexually differentiated *Limacina retroversa* in each month. Unshaded area represents hermaphroditic females, shaded areas hermaphroditic males. Area shaded with continuous lines refers to "population A," that with broken line, to "population B." ♂/♀ refers to the ratio of sex predominance in each month.

When the change in the percentage of the two sexual types among sexually differentiated *Limacina* from month to month as shown in Fig. 6 is calculated into ratios of sex predominance (the number of predominantly male individuals over those predominantly feminine) a change from a high value to unity is seen. These values are appended at the bottom of Fig. 6. It will be seen that in December and January this value is very high, but in May it is equal to one, while among the mixed populations the value is again in favor of the male type in the later part of the year.

In Fig. 7 are shown the proportions of "pure males," animals with little or no feminine tissue in the gonad, and "less than 25 per cent" male (very pronounced feminine) types among the sexually differentiated individuals in each month. About 45-55 per cent of sexually differentiated *Limacina* in December and January were "pure males," the rest being hermaphroditic males. In March the proportion of "pure males" was reduced greatly and from April onward this type of individual was not seen any more. On the other hand, pronounced feminine individuals with less than 25 per cent of the gonadal tissue made of male germ cells were not seen before May. It will also be noticed that

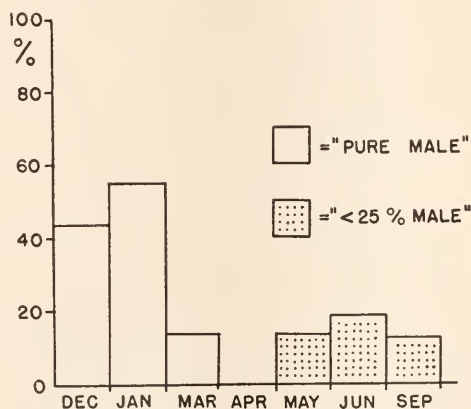


FIG. 7. Monthly appearance of "pure males" and hermaphroditic females with less than 25 per cent of their gonadal tissue consisting of male germ cells. Histograms before April refer to "population A" and those after April to "population B" with mixed populations. "Pure males" represented by unshaded areas; "less than 25 per cent male" type of hermaphroditic females represented by shaded areas.

the occurrence of this type of extreme female in the later part of the year has been always low in comparison to the other types.

Change in Reproductive Activity

The reproductive activities of *Limacina retroversa* show a parallelism with the morphological changes associated with the advance of time. During December and January when "pure" and hermaphroditic males constituted nearly all the groups of sexually differentiated individuals no hermaphroditic females with mature ova in the gonad were seen in the specimens examined, while spermatozoa, on the other hand, were evidenced as being liberated by the fact that mature male germ cells were

found in different parts of the gonoduct. It is in March, however, that individuals with mature ova in their ovotestes began to appear. This type of individual continued to increase in proportion until May. In May animals had been collected and fixed during their spawning activity. Later in the year all the different types of functional individual were seen. The occurrence of these types of functional individual in the various months is shown in Table IV.

TABLE IV

Distribution of functional types of *Limacina retroversa*, expressed in percentages, in different months.

1	2	3	4	5	6
Month	Number studied	Active males	Mature females	Spawning females	Functional hermaphrodites
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Dec.....	24	63	0	0	0
Jan.....	26	58	0	0	0
Mar.....	22	55	5	0	0
Apr.....	25	32	20	0	8
May.....	23	17	43	30	26
June.....	21	19	38	19	19
Sept.....	26	19	27	12	35

It will be seen from column 3 of this table that individuals ready to liberate spermatozoa are seen throughout the year, but that the proportion is higher in the earlier part of the year than in the later and that the reduction of the number of active males is accompanied by an increase in the number of individuals functioning as females (columns 4 and 5) and those acting as hermaphrodites (column 6) after April. A comparison of columns 4 and 5 will show that spawning is found after the mature females were observed in March. It has been found in the section on the relation between size and sexuality that about half of all the mature females examined were fixed during the act of spawning. From column 5 of Table IV it is seen that in May about three-quarters of the mature females were fixed in this stage, while in June the proportion of spawning females amounted to half of the matured ones, and in September, less than half. This indicates a higher spawning activity in May than in the other months. The fact that no spawning individuals were seen among the mature females in April does not exclude the possibility that egg-spawning took place in this month but that the individuals did not happen to be fixed with eggs in the gonoduct and selected for examination in this study. We may consider spawning to start be-

tween April and May and continue to the fall with May as the most important period for breeding.

From the above examination of the reproductive system of *Limacina* in terms of time it appears that among "population A," which entered the Gulf of Maine first, all the individuals were sexually differentiated by the beginning of March. In winter, a great majority, if not all, of them were "pure males." The number of such "pure males" decreased in March and they were not seen in April, giving place to individuals with a lesser degree of masculinity, to functional hermaphrodites and eventually to hermaphroditic females with very little masculine tissue in their gonads. The proportion of male tissue in comparison with the female in the gonad decreased as the animals grew. By April 40 per cent of the specimens collected had the feminine tissue so well developed as to warrant their being classified as hermaphroditic females. Animals with "less than 25 per cent male" gonads occurred after the "pure males" had disappeared from the collections. In May the two sexual types were equal in number. After May the bulk of the species in the Gulf was derived from the recently arrived "population B" which mixed with the descendants and remnants of "population A" and other new recruits. Among the mixed population sexually undifferentiated individuals were seen again and all the structural types were present.

The functional phases of reproduction are summarized in Fig. 8. More than half of the protandric males started to function in winter and the male phase continued into spring and summer as it became gradually replaced by the female and functional hermaphroditic phases of reproduction. Females began to attain maturation in March and the proportion of mature females increased rapidly in April and May. Spawning of ova commenced between April and May, being highest in the latter month. The largest proportion of functional individuals, including the male, female, and hermaphroditic types, is seen in May when half of them were spawners of eggs and the other half were males and hermaphrodites in function. These individuals were seen in the months from June to September also. It appears that May is the most important period for spawning though the reproductive activity was carried on into the autumn.

Change in Sex Predominance

A change in the value of the ratio of the sexes during growth is generally used as one of the indications of a change of sex. As the term "sex ratio" is not very appropriate for monoecious forms where both male and female reproductive tissues are present in the gonad,

it seems better to speak of sex predominance and ratio between male and female predominance. The decrease in the value of this ratio from small to large-sized animals may be due, in general, to any one of the following causes: (a) Sexual dimorphism such that the males are habitually smaller than the females, (b) differential mortality, that is, more males than females die as the animals grow, and (c) sex reversal or an actual change in the condition of the reproductive system as the animals grow. There is no evidence from our material either to show that there

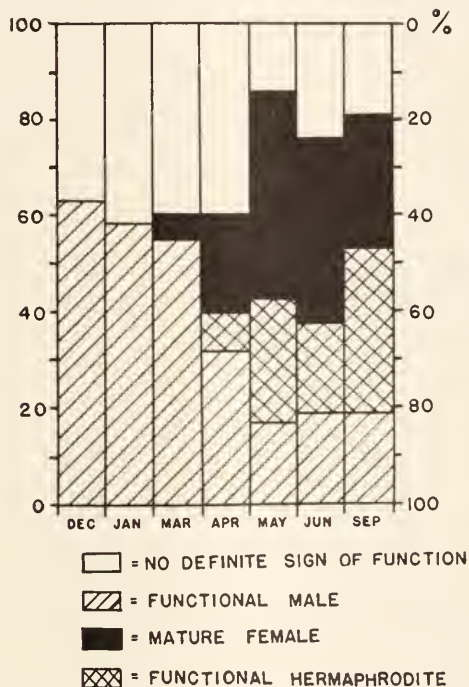


FIG. 8. Monthly distribution of the three functional types of *Limacina retroversa*.

is a sexual dimorphism or to indicate a differential mortality which may be interpreted as the cause of the appearance of a higher proportion of males among smaller-sized *Limacina* and a greater number of females among larger ones. The third alternative, that a change in the predominant sexuality takes place as the animals grow, however, has much evidence in its favor. In the first place, it has been shown above that there is a parallelism between the changes in the ratio of sex predominance with reference to size and that with reference to the advance of the season: male types dominate among smaller-sized *Limacina* and dur-

ing the winter months, and this excess of males decreases as the animals become larger and also as the season advances, and eventually the two types become equal among still larger-sized specimens and at the height of the breeding season in May. This parallelism indicates a change in the sexuality, both in structure and function, of the individuals as they grow.

In the second place, it has already been shown in a previous paper that there is no clear line of demarcation between male and female among *Limacina*, but that hermaphroditic male and female merge into each other and that a continuous series can be made out. As the size of the animals increases, a greater and greater portion of the gonad appears to contain female tissue. "Pure males" are confined to small-sized animals and in the winter months. The continuous change in the proportion of male and female reproductive tissues in the gonad culminating in many functional hermaphrodites and females with less than 25 per cent of the tissue of their sex glands made of masculine elements argues in favor of a reversal of sex predominance in this species. Furthermore, there is no "pure female" among *Limacina*: all the hermaphroditic females are large and apparently derived from male types.

Thirdly, a comparison of the size distribution of the different sexual types in the various months also shows that there is a change in sex predominance. In Fig. 9 the proportions of (a) sexually undifferentiated young, (b) individuals which are predominantly masculine and (c) those which are predominantly feminine, have been indicated in the histograms prepared by Redfield showing the distribution of size among all the specimens taken during each cruise. It shows the earlier occurrence of protandric males and later appearance, in March and April, of female types. The appearance of the female type among larger size groups suggests that they are transformed from the protandric individuals rather than from the very small undifferentiated ones.

From these morphological male and female types of *Limacina* functional hermaphrodites have also been indicated in the same figure (Fig. 9). These functional hermaphrodites increase from a very small proportion in April to a maximum in May and then decline in June. This change in the relative proportion of this type of individual supports the idea mentioned above that protandric males develop ovarian tissue while they are still functioning as males and eventually become functional females.

Finally, if the modal class of each month's collection is taken as representative of the total catch, the phenomenon of change of sex predominance can be brought out when the different modal size groups are compared. Analysis of the size frequency distribution showed that

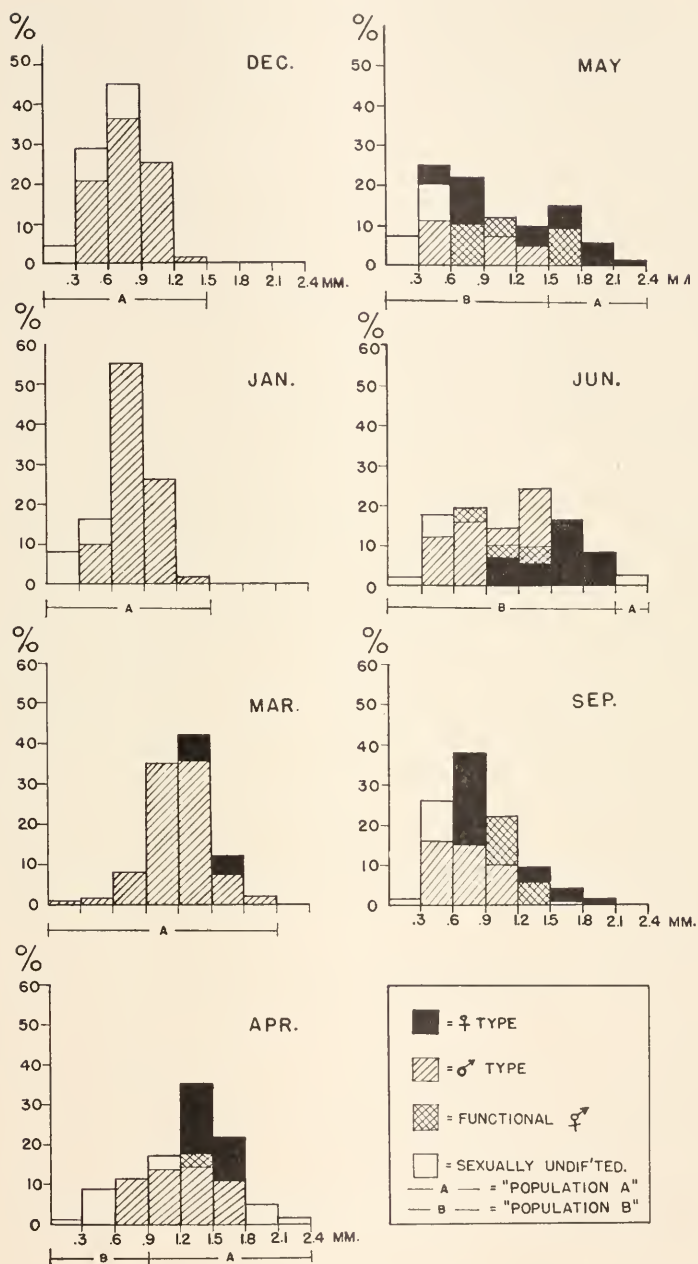


FIG. 9. Distribution of different types of *Limacina retroversa* among various size groups in each month. The proportion of each sexual type is added to the histogram showing size distribution prepared by Redfield (1939).

there is a single mode from December to April (Redfield, 1939). This modal class shifts from smaller to larger size during this period, showing a general growth of the members of the population. The sexual condition of the gonads of the modal class shows that not only growth, but sexual change has also taken place. In the modal class of the December material all the differentiated individuals are males and over 60 per cent of them have more than three-quarters of their gonadal tissues composed of male reproductive cells. Half of the males are ready to liberate spermatozoa. In January all the individuals of the modal class are predominantly male and 65 per cent of them are ready to discharge the product of their sex glands, thus showing an increase in the proportion of sexual differentiation with growth and an increase in the number of individuals capable of inseminating others. But it is in March that 11 per cent of the modal class are seen to have more female tissue in their gonads; the rest are still all predominantly male, half of which are ready to discharge or are discharging spermatozoa. The proportion of females increases to one-half of the modal class in April and some of the individuals begin to function both as male and female simultaneously. Of the two modal classes in May, the larger one shows, in the condition of the reproductive system, a continuation of the general changes of the previous months. For there is a further reduction of the male type and an increase of the functional hermaphrodites and actively functioning females. In this month, when the egg-laying individuals are added together, that is, spawning females plus functional hermaphrodites, they make up the whole of the modal class (see the right half of the histogram for May in Fig. 9). Thus if the modal class found by Redfield is taken as representative of the material collected, observations on the sexual condition of these groups give a consistent story of the change of sex predominance and function.

RELATION BETWEEN SEXUAL PHASES AND DISTRIBUTION

When the sexual condition of populations A and B are compared several interesting differences are seen. The first characteristic of the sexual phases of "population B" in contrast to "population A" is that sexual differentiation seems to commence earlier with reference to size. In "population A" individuals as large as 0.85 mm. in diameter have been seen as undifferentiated sexually, while the largest undifferentiated individual of "population B" is only 0.6 mm. in diameter. In addition to the earlier differentiation of the gonads, a second difference is seen in the manner of differentiation between the two populations. Instead of the male elements in the gonads always taking the lead in

development so as to bring about protandry, some individuals among "population B" have both types of sexual cells developed at nearly the same time in addition to protandry, so that proterogyny and hermaphroditism appear. In view of this type of development, small-sized mature hermaphroditic females and functional hermaphrodites may also be interpreted as derived directly from young undifferentiated individuals with the protandric stage omitted. In "population A" such a situation is not seen. The third point of difference is that the development of the female elements of the gonads of individuals in "population B" is also earlier. This can be seen when the minima of the size of the individuals which achieved maturation of ova in the two populations are compared. The minimal size of *Limacina* with mature ova is 0.8 mm. in diameter in April and 1 mm. in May among members of "population B," while among "population A" the minimal size of mature females is 1.3 mm. in April and 1.67 mm. in May. It is possible that these small mature females in "population B" did not pass through a protandric stage of noticeable length, though other specimens of the same population have a distinct young male phase preceding the female. In "population A" the smallest functional hermaphrodite is 1.5 mm. in diameter in April and 1.6 mm. in May, while during the same months functional hermaphrodites as small as 1.1 mm. in diameter are found among "population B."

In Fig. 9 the right portion of the May histogram which has been attributed to "population A" by Redfield is very similar to that of April in the distribution of sexual types. In both cases hermaphroditic females occupy the large size groups with functional hermaphrodites between them and the small-sized, predominantly male individuals. On the other hand, the left portion which represents "population B," has a size distribution comparable to those of the winter polygons but the sexual phases are quite different from them. Here the hermaphroditic females and functional hermaphrodites appear among protandric males in small size groups, which in the previous months only contain protandric and "pure" males.

All these differences in the sexual condition of the two populations seem to point convergently to one underlying factor, namely, that during the warmer part of the year sexual differentiation takes place sooner than in the colder time and the sexual phases may be telescoped together when differentiation is speeded up in the summer. This is in line with the previous observations made on *Ostrea* by authors on both sides of the Atlantic (Coe, 1932, 1936; Needler, 1932; Orton, 1926; Späreck, 1925). However, this does not exclude the possibility that there is a

difference in the origin of "populations A and B" which may account for some of the observed differences in sexual development.

COMPARISON OF THE REPRODUCTION OF *LIMACINA RETROVERSA* WITH OTHER MOLLUSKS

As the reproductive history of no other Opisthobranchiata has been described, it is not possible to compare the reproduction of *Limacina retroversa*^{*} with that of the members of the same genus or family or even order. Only in a general way can the sexual sequence of this form be compared with that found among other mollusks.

In the winter months *Limacina* which entered the Gulf of Maine are of small size and sexually undifferentiated. As they grow this initial phase is followed by protandry in which nearly all the individuals are predominantly male. The protandric phase is succeeded by a hermaphroditic phase during which the animals transform from a more masculine to a more feminine condition. In the final phase the individuals are predominantly female in structure and generally function as hermaphrodites, liberating both ova and spermatozoa, or as females only. This type of sexual transformation conforms in a general way with that found among gastropods. The sequence approximates most closely to that of the prosobranchiate gastropods such as *Valvata tricarinata* worked out by Furrow (1935) and *Patella vulgata* by Orton (1928). But the time required for the transformation of sex is different. Orton observed that the male *Patella* transformed into female at the age of one year, while Furrow found that in *Valvata* the different sexual functional phases followed each other very closely and the protandric phase was very short. However, in *Limacina retroversa* the change in sex predominance seems to take place during a period of two or three months. For instance, in one population which entered the Gulf of Maine in December and January, the protandric males were transformed into females from March to May. In the warmer part of the year this transformation required a shorter time for its completion.

SUMMARY

1. In the winter months sexual differentiation commences when the animals are between 0.6 and 0.85 mm. in diameter. In the summer sexual differentiation starts when the animals are smaller.

2. The early proliferation of the male reproductive cells leads to a protandric phase. The "pure males" give place to hermaphroditic males and later to hermaphroditic females as the size of the animals becomes greater than 1 mm. in diameter. After the specimens are 1.5

mm. in diameter the female reproductive cells overtake the male in development, bringing about a decrease in the proportion of hermaphroditic males and an increase of the opposite sexual type. This change continues until the two types are equal, when the animals are 2 mm. or more in diameter.

3. Functionally the male phase starts among smaller *Limacina* than do the functional female and hermaphroditic phases. The highest proportion of actively functioning males is found among animals 1.2 to 1.8 mm. in diameter. Among larger animals the functional hermaphroditic and functional female phases replace the male phase. Hermaphroditic females begin the functional phase after they are greater than 1 mm. in diameter.

4. Members of a population which entered the Gulf of Maine in the winter were all sexually differentiated by the beginning of March. Nearly all of these individuals developed into protandric males, about half of which were "pure males." The number of such "pure males" decreased in March and disappeared in April, being replaced by animals with greater predominance of feminine tissue in the gonads. By April about 40 per cent of the specimens were hermaphroditic females and in May the two sexual types were equal.

5. The male functional phase began in winter and continued into spring and summer. Hermaphroditic females matured in March and their proportion increased in April and May. Spawning of ova commenced by the end of April. The largest proportion of functional individuals of all types was seen in May.

6. Reasons are advanced for the belief that the change in sexual constitution of the populations is due to a reversal of sex predominance of the individuals.

7. The sequence of the sexual phases of *Limacina retroversa* agrees in a general way with those found for other members of the gastropod group of mollusks, particularly that of *Valvata*.

This study has been carried out under the helpful direction of Professor Redfield to whom the writer wishes to express his indebtedness.

BIBLIOGRAPHY

- AMEMIYA, I., 1929. On the sex change in the Japanese common oyster, *Ostrea gigas* Thunberg. *Proc. Imper. Acad. Tokyo*, 5: 284.
- AWAITI, P. R., AND H. S. RAI, 1931. *Ostrea cucullata* (Bombay oyster). *Indian Zool. Mem.*, 3: 1.
- BOUCHON-BRANDELY, M., 1882. On the sexuality of the common oyster (*O. edulis*) and that of the Portuguese oyster (*O. angulata*). Artificial fecundation of the Portuguese oyster. *Bull. U. S. Fish. Com.*, 2: 339.

- BURKENROAD, M. D., 1931. Sex in the Louisiana oyster, *Ostrea virginica*. *Science*, **74**: 71.
- COE, W. R., 1931. Sexual rhythm in the California oyster (*Ostrea lurida*). *Science*, **74**: 247.
- COE, W. R., 1932a. Sexual phases in the American oyster (*Ostrea virginica*). *Biol. Bull.*, **63**: 419.
- COE, W. R., 1932b. Development of the gonads and the sequence of the sexual phases in the California oyster (*Ostrea lurida*). *Bull. Scripps. Inst. Ocean. Tech. Ser.*, **3**: 119.
- COE, W. R., 1933. Sexual phases in *Teredo*. *Biol. Bull.*, **65**: 283.
- COE, W. R., 1934a. Alteration of sexuality in oysters. *Am. Nat.*, **68**: 236.
- COE, W. R., 1934b. Sexual rhythm in the pelecypod mollusk *Teredo*. *Science*, **80**: 192.
- COE, W. R., 1935a. Influence of external stimuli in changing the sexual phase in mollusks of the genus *Crepidula*. *Anat. Rec.*, Suppl., **64**: 80.
- COE, W. R., 1935b. Sequence of sexual phases in *Teredo*, *Ostrea* and *Crepidula*. *Anat. Rec.*, **64**: 81 (suppl.).
- COE, W. R., 1936a. Environment and sex in the oviparous oyster *Ostrea virginica*. *Biol. Bull.*, **71**: 353.
- COE, W. R., 1936b. Sequence of functional sexual phases in *Teredo*. *Biol. Bull.*, **71**: 122.
- COE, W. R., 1936c. Sexual phases in *Crepidula*. *Jour. Exper. Zool.*, **72**: 455.
- COE, W. R., 1936d. Sex ratio and sex changes in mollusks. Manifestation P. Pelseneer Bruxelle, p. 69.
- FURROW, C. L., 1935. Development of the hermaphrodite genital organs of *Valvata tricarinata*. *Zeitschr. f. Zellforsch.*, **22**: 282.
- GEMMILL, J. F., 1896. On some cases of hermaphroditism in the limpet (*Patella*) with observations regarding the influence of nutrition on sex in the limpet. *Anat. Anzeig.*, **12**: 392.
- GEMMILL, J. F., 1900. Some negative evidence regarding the influence of nutrition on sex. *Communicat. Millport. Mar. Biol. Stat.*, **1**: 32.
- GOULD, H. N., 1917. Studies on sex in the hermaphrodite mollusk *Crepidula plana*. II. Influence of environment on sex. *Jour. Exper. Zool.*, **23**: 225. III. Transference of the male producing stimulus through sea-water. *Jour. Exper. Zool.*, **29**: 113.
- GRAVE, B. H., AND J. SMITH, 1936. Sex inversion in *Teredo navalis* and its relation to sex ratios. *Biol. Bull.*, **70**: 332.
- GUTSELL, J. S., 1926. A hermaphroditic viviparous oyster on the Atlantic coast of North America. *Science*, **64**: 450.
- HSIAO, SIDNEY C. T., 1939. The reproductive system and spermatogenesis of *Limacina* (*Spiratella*) *retroversa* (Flem.). *Biol. Bull.*, **76**: 7.
- LOOSANOFF, V. L., 1937. Seasonal gonadal changes of adult clams, *Venus mercenaria*. *Biol. Bull.*, **72**: 406.
- NEEDLER, A. B., 1932a. Sex reversal in *Ostrea virginica*. *Can. Biol. and Fish.*, **7**: 283.
- NEEDLER, A. B., 1932b. American Atlantic oysters change their sex. *Prog. Rept. Atl. Biol. Sta. and Fish. Exp. Sta.*, **5**: 3.
- ORTON, J. H., 1909. On the occurrence of protandric hermaphroditism in the mollusc *Crepidula fornicata*. *Proc. Roy. Soc., Ser. B.* **81**: 468.
- ORTON, J. H., 1919. Sex-phenomena in the common limpet (*Patella vulgata*). *Nature*, **104**: 373.
- ORTON, J. H., 1922. The phenomena and conditions of sex-change in the oyster (*O. edulis*) and *Crepidula*. *Nature*, **110**: 212.
- ORTON, J. H., 1926. Observations and experiments on sex-change in the European oyster (*O. edulis*). *Jour. Mar. Biol. Ass'n*, **14**: 967.

- ORTON, J. H., 1928. Observations on *Patella vulgata*. *Jour. Mar. Biol. Ass'n*, **15**: 851.
- ORTON, J. H., 1931. Observations and experiments on sex-change in the European oyster (*O. edulis*). *Jour. Mar. Biol. Ass'n*, **17**: 315.
- PELSENEER, P., 1894. Hermaphroditism in Mollusca. *Quart. Jour. Micr. Soc.*, **37**: 19.
- PELSENEER, P., 1926. La proportion relative de sexes chez les animaux et particulièrement chez les mollusques. *Mém. Acad. Roy. Belg. Sci.*, **8**: 11.
- REDFIELD, ALFRED C., 1939. The history of a population of *Limacina retroversa* during its drift across the Gulf of Maine. *Biol. Bull.*, **76**: 26.
- SPÄRCK, R., 1925. Studies on the biology of the oyster. *Rept. Dan. Biol. Sta.*, **30**: 1.
- YOUNGE, C. M., 1926. Protandry in *Teredo norvegica*. *Quart. Jour. Micr. Soc.*, **70**: 391.



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NOTES ON THE FAUNA ABOVE MUD BOTTOMS IN DEEP WATER IN THE GULF OF MAINE¹

HENRY B. BIGELOW AND WILLIAM C. SCHROEDER

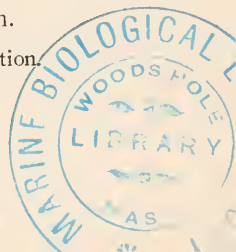
(From the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts)

INTRODUCTION

It has long been known that the waters just above bottom, in the mud-floored depressions of the western side of the Gulf of Maine at depths of 70-90 meters, are the home of an abundant population of the large edible shrimp, *Pandalus borealis*, accompanied by the smaller species, *P. montagui*, *P. propinquus* and *Dichelopandalus leptoceros* (Rathbun, 1883, p. 142; 1884, p. 819). After the introduction of the otter trawl to the Gulf of Maine waters, small amounts of these shrimps were landed from time to time in New England ports, and experimental trawling by the General Sea Foods Corporation in 1927-1928 again revealed the presence of *P. borealis* and its companion species in commercial quantities in the deep bowl just north of Cape Ann (Birdseye, 1928). But exploitation failed to follow, although Hjort's explorations of 1897 and 1898 (Hjort and Ruud, 1938) had long before led to the development of an extensive fishery in the other side of the Atlantic, which has gradually expanded to all suitable localities around the coasts of Norway.

Investigations by Hjort and Ruud (1938) and by Broch (1935) had also shown that *P. borealis* is but one member of a distinctive faunal community, some of the members of which live on bottom, others in the waters close above. Information from various sources had similarly shown that the *Pandalus* of the Gulf of Maine have as companions the same species of flat fishes, of rose fish (*Sebastes*), and of rockling (*Enchelyopus*), as in Norway, as well as several gadoids (*Merluccius*, *Urophycis*), which while peculiar to American waters, are at least similar to gadoid members of the Norwegian shrimp community. Hjort and Ruud's successive explorations had further demonstrated that *Pandalus* occurs in greatest abundance in situations where the bottom waters are sufficiently quiescent for organic debris to settle to the bottom.

¹ Contribution No. 210 from the Woods Hole Oceanographic Institution.



The presence of similar faunal assemblages living under conditions presumably the same in the two sides of the Atlantic opens interesting problems of environmental control. The Woods Hole Oceanographic Institution, therefore, welcomed the opportunity, in August, 1936, to make a further survey of the Gulf of Maine shrimp grounds with "Atlantis," under Professor Hjort's personal supervision, and with the assistance of Captain Hagbart Høium, an experienced Norwegian shrimp trawler.

The purpose of the investigation being primarily to explore the abundance of *Pandalus* and of associated fishes in a favorable environment, most of the hauls were made in the bowl north of Cape Ann and west of Jeffrey's Ledge (Area A, Fig. 1), where shrimps were already known to be plentiful, and which resembles the Norwegian shrimp grounds in its fjord-like topography and considerable depth. For comparative purposes, hauls were also made in the northeastern and southwestern branches of the open basin of the Gulf (Areas B, C, Fig. 1). Corings of the bottom for chemical analyses were obtained at thirteen of the stations.

The catches were so encouraging from the fisheries standpoint, that the exploration of the local shrimp stock was continued during the following two months by local fisheries interests—again under the supervision of Professor Hjort and of Captain Høium.

The present report on the regional distribution of the more prominent members of the *Pandalus* community is a sequel to Hjort and Ruud's (1938) preliminary report, and to Walford's (1936) discussion of the prospects of the shrimp fishery that appears now to be in the process of development.

Methods

The hauls (with two exceptions) were made with otter trawls of the sort used in the Norwegian shrimp fishery, brought by Professor Hjort from Norway for the purpose, the distinctive feature of which is that the footrope works about six inches above the bottom to avoid catching mud, with a sweep rope suspended below (for detailed description, see Walford, 1936). The success of the cruise was largely due to Captain Høium's skill in their use. One trial was also made with the footrope scraping the bottom. The mesh-size of the Norwegian nets was $1\frac{3}{4}$ inches (stretched) throughout. But four sizes of trawl were employed, their footropes respectively 35, 76, 82, and 117 feet in length, and the hauls varied in duration from 30 to 90 minutes, at an average speed of 1.5 knots. It has therefore been necessary to adjust the catches to a common standard, for comparative purposes,

a 60-minute haul with 82-foot trawl being chosen as the procedure most often followed during the cruise. Unfortunately, this adjustment is only a rough approximation, because the precise outline assumed by an otter trawl depends on a variety of factors such as speed, angle of the

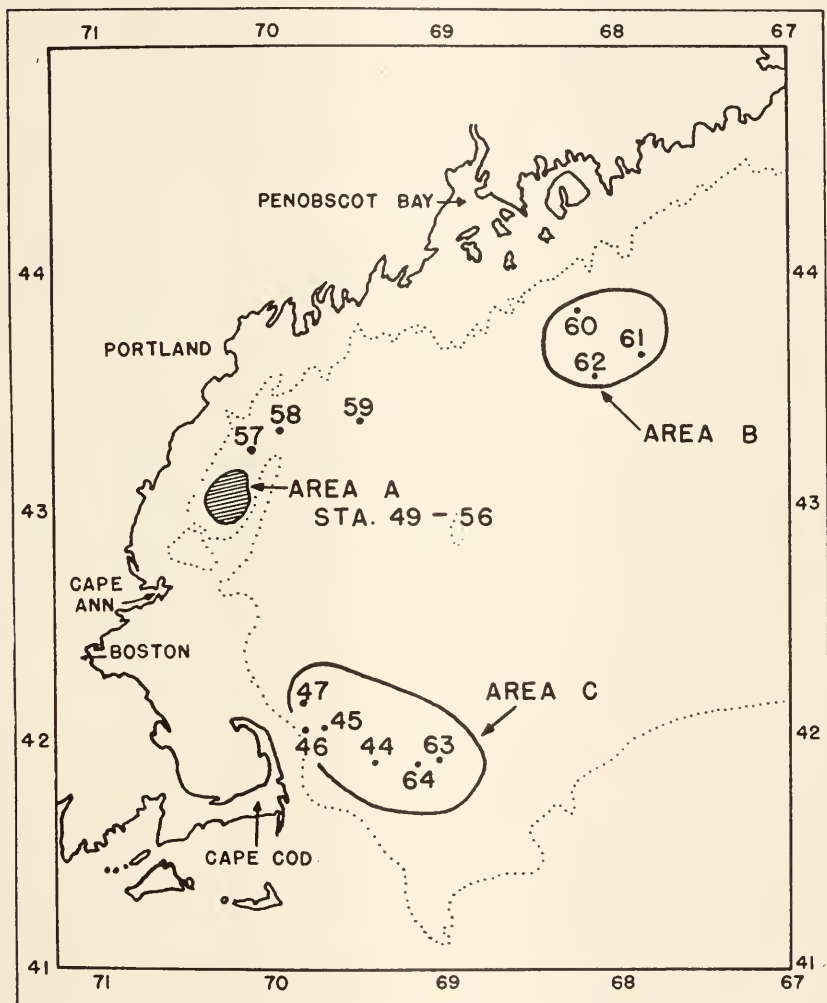


FIG. 1. Chart of western part of the Gulf of Maine, showing locations of "Atlantis" stations 2644-2664, August 18-23, 1936.

boards, and the type of bottom over which it is being drawn. The present calculation is based on the assumption that the breadths of sweep of trawls with footropes of different lengths are in the same ratios as semicircles of corresponding perimeters.

The chemical procedure, for which our thanks are due to Dr. S. A. Waksman, consisted of analyses of the upper half-inch of each core, for carbon, by the chromic acid oxidation method, and for total nitrogen by a semi-micro-Kjeldahl method. These were carried out at the New Jersey Agricultural Experiment Station at New Brunswick, New Jersey. Measurements were also made at Woods Hole of percentage loss on ignition, but showed considerable irregularity. Since this loss does not

TABLE I

Catches of the more prominent species at each station, adjusted as above (p. 306). Numbers italicized are derived from approximate counts.

Station	Depth	Amounts of Pandalus in liters	Numbers of Sebastes	Numbers of Merluccius	Numbers of Hippoglossoides	Numbers of Glyptocephalus	Numbers of Urophycis tenuis	Numbers of other fish†
2644	200	5	<i>108</i>	15	0	0	11	6
2645	183	2	16	8	0	2	3	4
2646	144	2	<i>432</i>	<i>240</i>	2	0	65	4
2647	228	1	3	1	0	0	7	11
2649	164	32	150	<i>700</i>	12	10	32	2
2650*	175	24	<i>224</i>	338	4	1	36	0
2651*	168	11	<i>131</i>	<i>262</i>	3	2	6	1
2652	160	71	<i>168</i>	<i>196</i>	13	14	39	1
2653†	155	168	107	20	47	24	28	34
2654	158	126	183	<i>840</i>	13	35	11	2
2655	168	100	<i>150</i>	<i>225</i>	13	3	40	2
2656	177	56	<i>283</i>	<i>351</i>	63	12	23	7
2657a	120	2	<i>100</i>	<i>160</i>	5	0	20	1
2657b	120	3	<i>103</i>	67	17	8	40	2
2658a	162	16	<i>400</i>	<i>467</i>	9	1	67	4
2658b	163	50	<i>450</i>	<i>600</i>	10	5	0	3
2659a	164	48	<i>150</i>	<i>600</i>	24	12	50	6
2659b	164	27	<i>188</i>	<i>700</i>	13	9	70	3
2660	192	1	250	300	26	18	100	14
2662	198	1	270	100	0	2	0	5
2663	183	4	<i>833</i>	<i>200</i>	5	0	47	39
2664	215	1	67	47	0	0	12	7

* Net damaged, calculations assume that only half the catch was saved.

† In this haul, the footrope scraped the bottom.

‡ See Table II.

represent the organic fraction alone, but also such water as is driven off from the clay fraction at high temperatures, these values are not included in the present discussion.

Qualitative Composition of the Community

The most interesting aspects of the catches from the qualitative standpoint are that the number of species taken in abundance at any given station was so small (Table I), and that the same few species

dominated, in all three areas visited, as well as at intervening locations. Among the catches of shrimps, for example, only occasional specimens of other related species, or of *Pasiphaea*, were noted among the more plentiful *P. borealis*. In fact, the latter so predominates among the shrimp population in deep water in the Gulf that a sample taken at random from catches made at depths greater than 160 meters in the Jeffrey's Bowl, in the summer of 1938, consisted of 738 specimens of *P. borealis*, but only 9 of all other pandalids combined. In shoaler water, however, in that general vicinity, the relationship is the reverse, as off Casco Bay in 30-40 meters, and at a neighboring station in about 90 meters, where *P. propinquus* greatly predominated over *P. borealis*.

TABLE II

List of "various" fishes other than those recorded in Table I, trawled on bottom in Jeffrey's Bowl and in the open basin of the Gulf of Maine, during August, 1936.

Species	Jeffrey's Bowl	Open basin
<i>Myxine glutinosa</i>	x	x
<i>Squalus acanthias</i>	x	x
<i>Raia senta</i>	x	
<i>Raia stabuliforis</i>	x	x
<i>Raia scabrata</i>	x	x
<i>Clupea harengus</i>	x	x
<i>Artediellus uncinatus</i>	x	
<i>Cyclopterus lumpus</i>	x	
<i>Lumpenus lampetraeformis</i>	x	
<i>Cryptacanthodes maculatus</i>	x	x
<i>Pollachius virens</i>		x
<i>Gadus callarias</i>	x	x
<i>Melanogrammus aeglefinus</i>		x
<i>Urophycis chuss</i>	x	x
<i>Urophycis chesteri</i>		x
<i>Enchelyopus cimbrius</i>	x	x
<i>Brosme brosme</i>		x
<i>Macrourus bairdii</i>		x
<i>Lophius americanus</i>		x

(39 to 0 and 39 to 1 respectively) at that same time. This is in line with Rathbun's (1883, p. 143) statement that while *P. montagu* and *Dichelopandalus leptoceros* are often associated with *P. borealis* over soft bottoms, they are not only the most plentiful in shoaler water, but occur on gravelly and sandy bottoms as well as on mud.

Our catches of fishes, in August, 1936, were similarly dominated by a group of 5 species, in combination, namely: by rose fish (*Sebastes marinus*), silver hake (*Merluccius bilinearis*), hake (*Urophycis tenuis*) and flat fishes (*Glyptocephalus cynoglossus* and *Hippoglossoides platessoides*), and to such an extent that the total catch of about 11,900 fish



included only 183 specimens of all the other species combined, that are included in Table II. The total list of 24 species together with a few others that have been picked up in deep trawlings previously (*Argentina*, *Triglops*, *Cottunculus*, *Aspidophoroides*, *Liparis*, *Pholis*, *Leptoclinus*, *Zoarces*, *Lycenchelys*) includes the great majority of the members of the Gulf of Maine fish fauna that are to be expected on the floor of the deep troughs, whether as regular inhabitants or stragglers. This is a very much less varied assemblage than inhabits the corresponding depth zone along the offshore slope of the Gulf of Maine and of southern New England (for complete list of records there, see Goode and Bean, 1896).

Regional Distribution

Regional segregation of the catches (Table III) shows that shrimps were taken in much greater average abundance in the deepest part of the Jeffrey's Bowl (Area A) and on the northern slope of its northern rim (Station 2658, 163 meters) than in either branch of the main basin of the Gulf (Areas B, C).

TABLE III

Regional distribution of average catches.

Area	Hauls	Shrimp	Sebastes	Mer-lucius	Flat fish	Uro-phycis	Various fishes	Total fishes
	Number	Liters	Numbers	Numbers	Numbers	Numbers	Numbers	Numbers
A	7	60	187	416	28	26	2	649
B	2	1	260	200	23	50	10	543
C	6	2	243	92	2	34	12	383
St. 2658	2	33	425	534	13	0	5	977

From the environmental standpoint, the outstanding feature of this Bowl is the fjord-like character given to its deepest parts (170–192 meters), by the shallow ridge of Jeffrey's Ledge to the south and offshore, and on the north, by a sill with a maximum depth of about 134–137 meters (Fig. 2). This enclosure appears to be more effective, so far as concerns the activity of circulation of the bottom waters, than the topography of the sill might suggest, for temperature averages some 1–2° colder in midsummer in the bottom of the trough than at corresponding depths in the open basin to the east of the barrier, only a few miles distant.

Hjort and Ruud (1938, p. 103) have already pointed out that the mud was richest in carbon and in nitrogen where large catches of shrimps were made. This is corroborated by the following comparisons of the catch of shrimps with the percentages of organic carbon and of nitrogen

in the corings (Table IV), the average being 4.02 per cent of organic matter for the group of stations where the catch of shrimps was more than 30 liters, but only 3.05 per cent for stations yielding 5 liters of shrimps, or less.

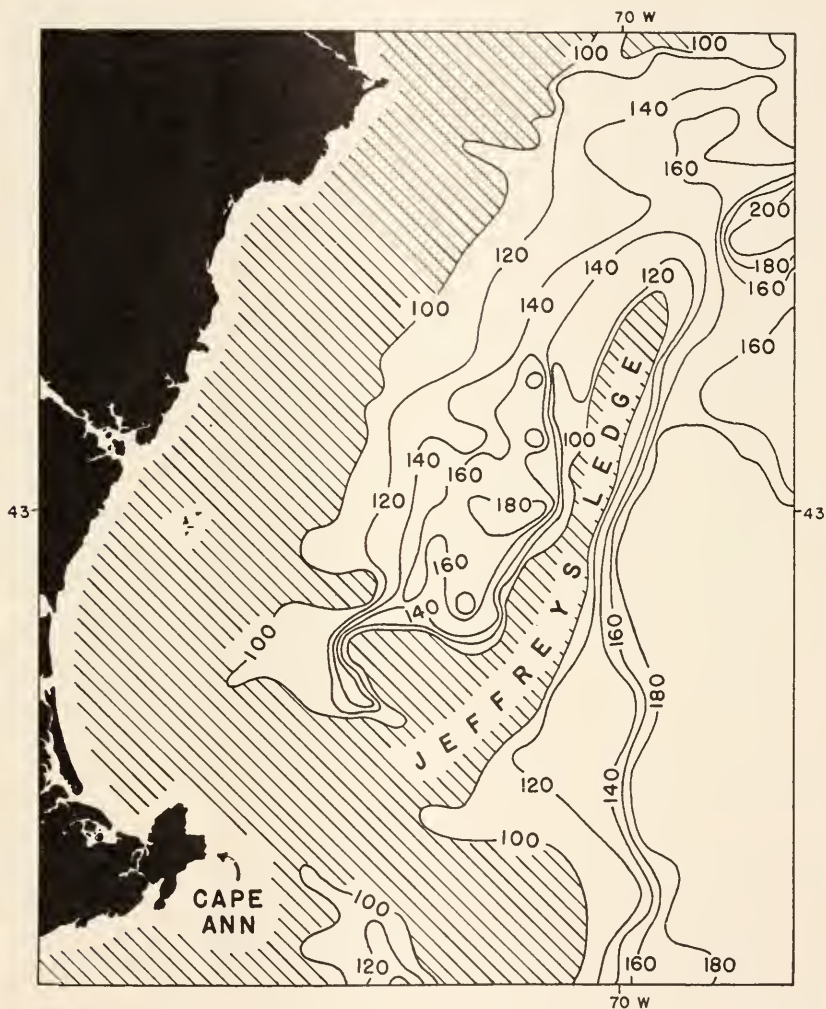


FIG. 2. Submarine topography of the region between Jeffrey's Ledge and the western coast-line of the Gulf of Maine at depths greater than 100 meters.

At one station (2662), it is true, where the percentage of organic matter was high, the catch of shrimps was very small; regional irregularities are, in fact, to be expected, in the nature of the case. But in no instance was the mud poor in organic content where shrimps were

abundant. While the correlation is not so close between the catches of shrimps and the percentage of nitrogen, the latter averaged 0.338 per cent for the rich and 0.234 per cent for the poor shrimp stations. In other words, the average percentage of organic carbon was about one-third higher, and that of nitrogen about one-half higher where catches of shrimp were relatively large than where they were small. Unfortunately, the data do not afford so satisfactory a comparison from the regional standpoint, because the mud was sampled at only two of the stations in the deeper part of the Jeffrey's Bowl. At each of these (Stations 2649, 2654), however, the percentage of organic carbon closely approached the maximum found at any station, while the percentages of nitrogen (0.295 and 0.309 per cent) were at least somewhat above the

TABLE IV

Percentage of organic matter and of nitrogen in dry mud and catch of *Pandalus*.

Station	Shrimps	Organic matter*	Nitrogen
	<i>liters</i>	<i>per cent</i>	<i>per cent</i>
2654	126	4.1	0.309
2659	37 av.	3.7	0.195
2658	33 av.	4.2	0.553
2649	32	4.1	0.295
2644	5	2.6	0.311
2663	4	2.6	0.115
2657	2.5 av.	2.4	0.201
2645	2	3.7	0.217
2647	1	3.1	0.205
2660	1	3.3	0.301
2662	1	4.0	0.310†
2664	1	2.8	0.217

* Carbon X 1.724.

† Determination made at Woods Hole.

average for the series as a whole (0.262 per cent). It is interesting that the station that was the richest of all, both in organic carbon and nitrogen (Station 2658) was at least moderately productive of shrimps, though located north of the sill. Thus, it seems sufficiently established, both for Norwegian waters and for the Gulf of Maine, that in summer *Pandalus* is abundant only in situations where organic matter is plentiful on the sea floor below; and by available evidence this applies to the males throughout the year. But the fact that considerable catches of ovigerous individuals were landed by fishermen from depths of 70-90 meters only, in the northwestern side of the Gulf, during the winter of 1938 suggests that the large females tend to work inshore into shoaler and more turbulent situations during the egg-bearing period.

It seems that we are dealing here with a food relationship, for while no direct observations are available as to the diet of *Pandalus* in the Gulf of Maine, Wollebaeck (1903, see also Hjort and Ruud, 1938, p. 60) found the stomachs of this shrimp in Norwegian waters to contain animal remains of various origins, among a mass of indeterminate mud, evidence that while it feeds on bottom, its diet consists of the remnants of dead organisms or perhaps of bacteria and protozoa, rather than of the mud itself. This is in line with Waksman's (1933) demonstration that the organic matter in the mud is largely in the form of humus, and hence not likely to be readily digestible by the higher animals.

The catches of *Merluccius* also averaged considerably larger in Area A and at the neighboring station (2658) than in either of the other areas, though there is no reason to suppose that the type of bottom is of direct influence in this case, but rather that the significant parallelism is between the number of fish and of shrimps, as discussed below. *Sebastes*, on the other hand, averaged somewhat less plentiful in the Jeffrey's Bowl (Area A) than over the rim of the latter (Station 2658) or in the open basin (Areas B and C). But the difference in average catch from area to area was so small for pleuronectids and for *Urophycis* that it may not have been regionally significant.

Among the fishes of minor numerical importance, *Enchelyopus cimbrius* and *Urophycis chuss* were encountered at so many stations (respectively 13 and 12 hauls out of 22), that they appear practically universal over the deep bottoms both of the Jeffrey's Bowl and of the western branch of the open basin of the Gulf, though the maximum catch was only 9 specimens in the one case, 10 in the other. *Enchelyopus* was also taken in the eastern basin, and failure to take *U. chuss* there in our few hauls is no evidence that it does not actually occur there.

Odd specimens of all other species included in Table II, and listed on page 309, have also been taken in the bottom of the Jeffrey's Bowl at depths greater than 160 meters on one occasion or another, with the exceptions of *Triglops*, *Argentina*, *Aspidophoroides*, *Pholis*, *Leptoclinus*, *Brosme*, *Urophycis chesteri*, *Macrourus*, *Lophius*, haddock and American pollock. These are also to be expected there, either because plentiful in somewhat shoaler water in the near vicinity, or because already known to occur in deep water elsewhere in the Gulf. Arctic species in particular, such as *Cottunculus*, would be as likely to maintain a small population in the low temperatures of the Bowl as anywhere else to the west of Cape Sable.

In short, there is nothing in the available data to suggest that the sill responsible for the fjord-like character of this Bowl gives any distinctive character to the fish fauna of its bottom waters, as contrasted with

that of the open basin of the Gulf to the eastward. At most, certain species that have their center of abundance over the slope outside the Gulf (*Macrourus*, *Urophycis chesteri*, *Argentina*) may be progressively less numerous westward, until they reach the trough only as stragglers, if at all; whereas certain others may be most plentiful there, whether because of cold water affinity, or to utilize the particular food supply provided by the local abundance of *Pandalus*, as appears to be the case with *Merluccius*.

Urophycis tenuis has long been known to feed chiefly on shrimp (see Bigelow and Welsh, 1925, p. 450, for summary of its feeding habits); in fact, all but one of the specimens of this species that were opened during our cruise contained more or less remnants of large *Pandalus*. But the following tabulation (Table V) fails to suggest any

TABLE V
Numerical relationship of fish to shrimps.

Shrimps, liters	126	100	71	56	54	48	32	27	24	16	11	5
<i>U. tenuis</i> , numbers	11	30	40	26	0	50	34	75	36	67	6	23
<i>Sebastes</i> , numbers	183	150	168	283	450	150	150	188	224	400	131	108
Flat fish, 2 sp., numbers . .	48	16	25	75	15	36	22	22	6	10	5	0
<i>Merluccius</i> , numbers	840	225	196	351	600	600	700	700	338	467	262	15

Shrimps, liters	4	3	2	2	2	1	1	1	1
<i>U. tenuis</i> , numbers	60	40	4	86	20	14	100	0	19
<i>Sebastes</i> , numbers	833	103	16	432	100	3	250	270	67
Flat fish, 2 sp., numbers . .	5	25	2	2	5	44	2	2	0
<i>Merluccius</i> , numbers	200	67	8	240	160	1	300	100	47

correlation between the numbers of *Urophycis* and the amounts of shrimp taken, for the average catch of the former averaged about the same for the nine stations which yielded 24 liters or more of shrimps, as for the ten stations that yielded 5 liters or less (34 and 37 *Urophycis*, respectively), while the largest and the smallest catches of *Urophycis* were each made at a station where shrimps were very scarce.

The catches of *Sebastes* seem also to have been independent of those of shrimps, the average being almost exactly the same for the group of stations where shrimps were plentiful (20 liters or more), as for the

group where they were scarce (216 and 218 *Sebastes*, respectively); while the largest and smallest catches of *Sebastes* (833 and 3) were both made at stations yielding only 4 liters and only 1 liter of shrimps, respectively. Neither do the present data yield any information as to the extent to which *Pandalus*, when abundant, serves as prey for *Sebastes*, the great majority of the latter having regurgitated the stomach contents before they were brought on board.

A rough parallelism does, however, appear, between the abundance of *Merluccius* and of shrimps, illustrated by the facts that the catch of the former averaged four times as great (494) for the group of stations

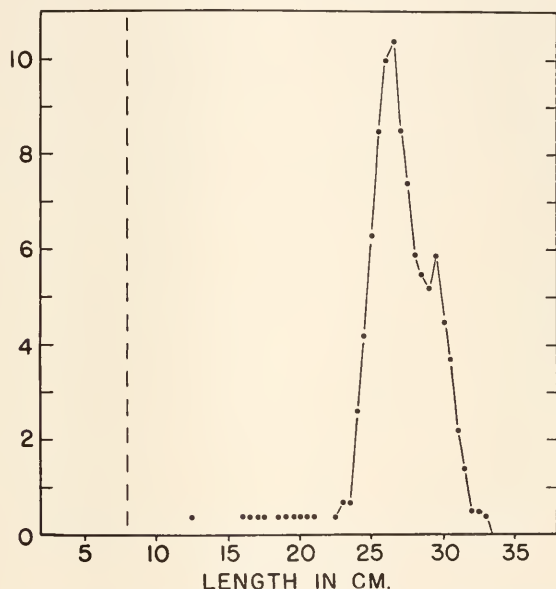


FIG. 3. Length-frequency distribution of 270 specimens of *Sebastes*, Station 2662, smoothed once by running average of three. The broken curve marks the lower limit for adequate sampling by the nets employed.

where shrimps were abundant as where they were scarce (114); that it was only where the catch of shrimps was less than 6 liters that the catch of *Merluccius* was as small as 100; and that no hauls where shrimps were so scarce yielded as many *Merluccius* as the average for the series as a whole (306). To judge from the predaceous nature of this fish and from its independence of the bottom, we are dealing here with a predator-prey relationship, though our present data fail to yield direct information in this regard for *Merluccius* any more than for *Sebastes*, and for the same reason.

Flat fishes of the two species, combined, also averaged considerably

more plentiful (av. 30 flat fish) where shrimps were in more than moderate abundance than where the catches of shrimps were 5 liters or less (av. 9 flat fish). But there being no reason to suppose that either of these pleuronectids preys to any extent on shrimps, the parallelism in this case suggests that the same muddy bottoms which provide the richest feeding for *Pandalus* are also rich in the small invertebrates on which *Hippoglossoides* and *Glyptocephalus* chiefly subsist. While we have no precise data, in this respect, for the Gulf of Maine, such is certainly true in Norwegian waters, where soft bottoms in similar situations

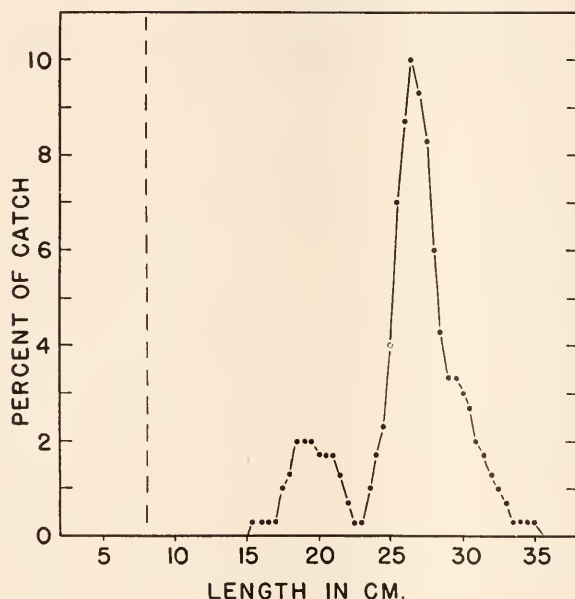


FIG. 4. Length-frequency distribution of 300 specimens of *Sebastes*, Station 2663, smoothed once by running average of three. The broken curve marks the lower limit for adequate sampling by the nets employed.

support a wealth of invertebrate life (Broch, 1935; see also Hjort and Ruud, 1938, p. 106).

Size Distribution of Different Species of Fish

It was likely due to the mesh-size of our trawls that the catches of *Pandalus borealis* consisted almost wholly of medium and large-sized individuals, the commonest lengths being from 12 to 18 cm. (rostrum-telson), which covers the upper size range for that species. But this factor cannot be invoked to explain a great scarcity, in the catches of *Sebastes*, of *Urophycis*, and of the two common flat fishes, of the smaller sizes that the mesh might be expected to sample.

In the case of *Sebastes*, the great majority were upwards of 22–23 cm. long, averaging something like 25–30 cm., a size that would be classified as “medium to large” in statistics of the commercial fishery (Figs. 3, 4). In fact, out of a total of 4,334 specimens taken, only 115 were less than 20 cm. long, and only 56 smaller than 15 cm., although the net should certainly have sampled this spiny species adequately down to 7–9 cm., and have taken at least a scattering of still smaller sizes had they been present in numbers at all approaching those of the larger fish. The rate of growth of *Sebastes* in the Gulf of Maine has not been studied, but its pelagic larvae have been taken there up to a length of 2.1 cm. (to a considerably larger size in European waters). The facts that fry of 3.7 to 7.5 cm. are abundant on bottom in spring and summer (Bigelow and Welsh, 1925; Bigelow and Schroeder, 1936) and that in a collection made in summer at Eastport, Maine, many years ago, there is a distinct size group of 5.1–6.3 cm., make it likely that the peak at 7–9 cm. shown on Fig. 5 represents fish one year old, and the

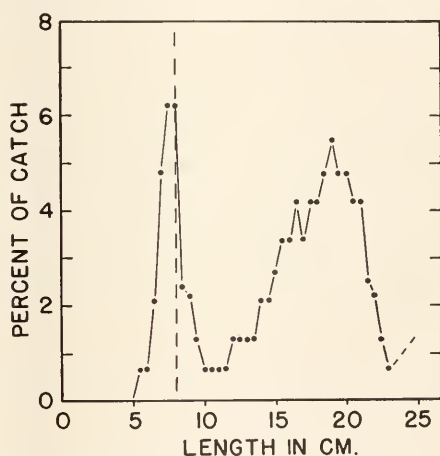


FIG. 5. Length-frequency distribution of all *Sebastes* taken less than 24 cm. long (146 specimens), smoothed once by running average of three. The broken curve marks the lower limit for adequate sampling by the nets employed.

next larger group centering at 15–21 cm., those two years old. On this basis, the fish of 24 cm. and larger, that formed the bulk of the catch in all three areas, were at least three years old and upwards. Unfortunately, our data afford no information as to how many year classes were actually included, for these older fish appear as a single size-group on the graphs for the two stations where unselected samples were measured (Figs. 3, 4, Areas B, C), while inspection of the fish larger than 20 cm. in Area A, as brought on deck, showed that there was no obvious succession of recognizable size-groups among them, either. In other

words, it is likely that had the entire catch of more than 4,000 specimens been measured, the resultant graph would have closely resembled Figs. 3, 4. However, since the dominant group includes the sizes that yield commercial catches, we may assume that the greater part of the usual life of the species is included from three years onward—however long that may endure. In fact, occasional specimens of 43–45 cm. are as close an approach to the maximum yet recorded for the Gulf (about 60 cm., Bigelow and Welsh, 1925, p. 305) as was to be expected from so few hauls.

The absence of small sizes is still more striking for *Urophycis tenuis*, for not a single specimen shorter than 21 cm. was taken among a total of 664, ranging up to about 70 cm. in length and to 8 lb. in weight, though the net could be depended upon to take them down to 16–17 cm. Again, in the cases of *Hippoglossoides* and *Glyptocephalus*, the smallest sizes (within the sampling range of the nets, or down to 10–15 cm.)

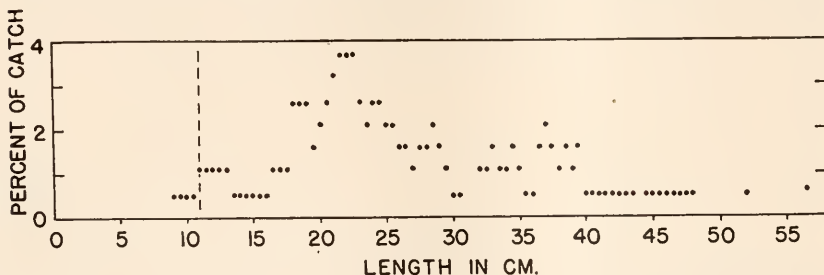


FIG. 6. Length-frequency distribution of 187 specimens of *Hippoglossoides platessoides*, smoothed once by running average of three. The broken curve marks the lower limit for adequate sampling by the nets employed.

were much less strongly represented than the successively larger sizes (Figs. 6, 7). In fact, *Hippoglossoides* yielded only 21 specimens shorter than 20 cm., and *Glyptocephalus* 17, out of totals of 183 and 135 respectively. If previous estimates of the rates of growth of these two pleuronectids in the Gulf of Maine be correct (Bigelow and Welsh, 1925, pp. 486 and 515; Bigelow and Schroeder, 1936, pp. 340–341), the first size-group of each that was well represented in the catches, centering at about 17–20 cm. for *Glyptocephalus* and at 20–23 cm. for *Hippoglossoides*, was at least two years old—with the yearlings only about one-fourth to one-third as numerous. Or, if Molander's (1925) estimate, based, however, on conditions in the Baltic, applies, the first prominent group of *Glyptocephalus* would be the four-year-olds. The evidence afforded by these two species is especially instructive, because the generality of distribution over the mid-range of included sizes shows that the local stocks had not recently been subject to any outstanding variations in the success of individual year classes.

In short, our catches contain a very much weaker representation of the first two or more year classes that the nets might be expected adequately to sample of *Sebastes*, of *Urophycis*, and of the two pleuronectids than of the older fish, whereas the reverse would be the expectation in a normal population distribution. Since this applies to four species of widely separated taxonomic groups, differing widely, too, in their life histories, it is safe to assume that we have to do with a general depth or regional relationship, not with a chance irregularity in the relative strengths of individual year classes, such as might have been argued had only one species been at issue. If, then, the year 1936 be acceptable as representative, it appears that this group of fishes does not disperse into the deep troughs of the Gulf until they are at least two or more

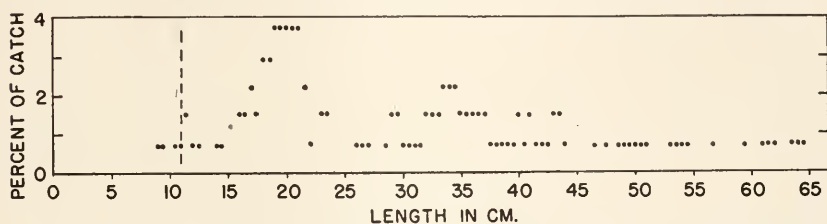


FIG. 7. Length-frequency distribution of 135 specimens of *Glyptocephalus cynoglossus*, smoothed once by running average of three. The broken curve marks the lower limit, for adequate sampling by the nets employed.

years old, a conclusion at least compatible with their generally non-migratory nature. The graphs further suggest that such individuals of these species as do stray out into these situations, or descend thither from the upper strata, tend, on the whole, to remain there year after year—or at least that none of these species shows any general tendency to depart thence, with increasing age. This appears also to apply to the few cod that wander out onto these deep soft bottoms, where very large specimens are sometimes caught, but very few small ones.

In the case of *Merluccius*, however, the catches were strongly dominated by a size group, with a modal length of about 17 cm., with the next longer (and very much less abundant) group centering at about 26 cm. (Fig. 8). Earlier studies (Bigelow and Schroeder, 1936, p. 338) would suggest that the first of these groups represents the fish hatched in the preceding summer, i.e., the yearlings, the second size group being the two-year-olds. Confirmation of this growth schedule for these comparatively cold waters is afforded by the fact that a small lot (28 specimens) taken near Mt. Desert in July, 1930, fell into the same approximate size divisions (15 fish, average, 15 cm.; 13 fish, average, 26 cm.) with no fish between 20 and 23 cm. in length. *Merluccius* of 18–21 cm. taken near Chatham in the same season may best be interpreted as

one-year-olds that had grown faster at somewhat higher temperatures. This leaves only the post larvae of the year unaccounted for, among the younger fish. These would be so small (perhaps 2.5–3.5 cm.) in the month in question—if indeed they descend into the bottom waters by that date—that our nets would not have taken them; nor would have until after several months more growth on their part, to judge from the fact that fry trawled by “Albatross II,” off Long Beach, New York, in 1928 were only 5.7–11.8 cm. long in November. Thus, *Merluccius* (unlike its companion species) passes its first summer of rapid growth (second summer of life) in great abundance in deep water. The dis-

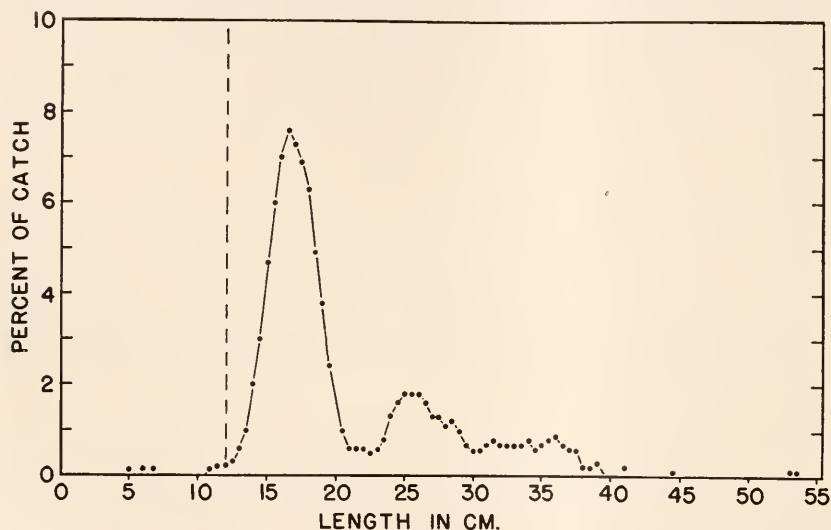


FIG. 8. Length-frequency distribution of 1,088 specimens of *Merluccius lili-nearis*, smoothed once by running average of three. The broken curve marks the lower limit, for adequate sampling by the nets employed.

covery of this habitat for it, combined with captures of this same size group in 18–22 meters near Mt. Desert, and of fish slightly larger in 45–50 meters off Cape Cod (mentioned above), is especially interesting, because previously this age group was close to unknown in the Gulf of Maine, although larvae are produced there in profusion, and larger fish of 20 cm. and upwards occur there in great abundance, both inshore and on the Banks (Bigelow and Welsh, 1925, p. 389). Present findings thus corroborate the earlier suggestion that young *Merluccius* are, in fact, as plentiful in the Gulf as the local abundance of adults would suggest, but that they keep to somewhat deeper water for the most part.

It appears, however, that most of the juvenile *Merluccius* that find their way out, or down, into the troughs, remain there through one summer of growth only, else our hauls might have been expected to

have yielded a much larger proportion of the next larger, and of subsequent size groups than was actually the case. Whether their subsequent departure takes place in the following autumn as part of the migration out of the Gulf of Maine, with which the silver hake is generally credited (Bigelow and Welsh, 1925, p. 388), or whether the troughs actually serve as wintering grounds for at least a part of the Gulf of Maine stock, must remain an open question, until these localities are explored during the cold months. We may, however, point out that the latter alternative would at least be compatible with the temperature cycle. The water at 150–170 meters is coldest in spring and summer, both in the Jeffrey's Bowl ($2.5\text{--}4.5^\circ$) and in the western branch of the western basin ($4.5\text{--}6^\circ$), warmest in late autumn or early winter ($5\text{--}7^\circ$ and $6\text{--}8^\circ$, respectively), while deeper still, in the basin the seasonal range is normally less than 1.0° (see Bigelow, 1927, Figs. 5, 6, 70, 72).

In any case, it now seems established that in subsequent feeding seasons most of the *Merluccius* that inhabit or visit the Gulf, repair to shoaler waters either on the Banks or around the coastal slope, to prey on the young fry of various other fishes.

Amounts of Pandalus and Fish Combined

Finally, speculation is perhaps permissible as to the total mass of animals, of a size our nets would sample, that exists in summer in the water next to bottom, in the rich shrimp area in the Jeffrey's Bowl. In this connection we have first to consider how much (if at all) the catches were impoverished by the failure of the nets to strain the water immediately in contact with the bottom. Our only direct evidence in this respect is the difference between catches in the standard hauls made with raised footrope, and in the one haul in which the footrope swept bottom (Station 2653), which yielded at the rate (adjusted) of 107 *Sebastes*, 20 *Merluccius*, 71 flat fish, 33 *Urophycis*, 34 "various" fishes, and 168 liters of *Pandalus*. This contrasts with an average of 377 *Sebastes*, 713 *Merluccius*, 32 flat fish, 49 *Urophycis*, 7 "various" fishes, and 99 liters of shrimps, for the three most productive catches of each of these categories with raised footrope, and with a maximum of 450 *Sebastes*, 846 *Merluccius*, 75 flat fish, 86 *Urophycis*, 11 "various" fishes, and 126 liters of shrimps for any one haul of the latter type in the same general area (Area A, Fig. 1). On this basis, it appears that inclusion of the six inches of water next the bottom might well have raised the average catch of shrimps in this region (the richest shrimp ground) by anywhere from one-third to two-thirds. Some increase in the catch of flat fish might also have resulted; and this last is made the more probable by the fact that Birdseye's (1928) twelve experimental hauls in the same region made with trawls of the usual pattern (footrope on bottom)

yielded a somewhat greater weight of large flat fish of the two species in question (average, 275 lb.) than of shrimps (average, 217 lb.), whereas the reverse was true² for our hauls with raised footrope. But there is nothing to suggest that the catches of the more active and more plentiful fishes were significantly reduced by our failure to sample the few inches of water that is in immediate contact with the sea floor. If anything the reverse is suggested, perhaps because the stirring of the mud by the trawl gives the fish warning of its approach. And these three species—*Merluccius*, *Sebastes*, and *Urophycis*—made up the major weight of fishes caught.

Assuming that, by actually sweeping the bottom, the average catch would have been increased by 50 per cent for shrimp, and by 30 per cent for flat fishes of the two species combined, but that the catches of the other major species of fish would not have been materially affected, the average for the Jeffrey's Bowl, deeper than 150 meters would be 37 flat fish, 187 *Sebastes*, 416 *Merluccius*, 26 *Urophycis*, 2 odd fishes, and 90 liters of *Pandalus*. Crediting the latter with an average weight of 0.97 lb. per liter as determined by measurements made on board, and using conversion factors of 1 lb. per fish for *Sebastes*, 0.25 lb. for *Merluccius*, 1 lb. for each of the pleuronectids, and 6 lb. for *Urophycis tenuis*, as derived from examination of the catches, and perhaps 1 lb. for the odd fishes of other species, we arrive at a general average weight of some 576 lb. per standard haul, for a water layer perhaps two meters in thickness. This corresponds to 55 lb. of fish and shrimps, above each acre, on the assumption that the breadth of the strip fished by the 82-foot trawl was about 52 feet, and that the distance covered averaged 1.5 sea miles per haul.

We should emphasize that if the foregoing assumptions even approximate the truth, the resultant weight is minimal, because it is based on the further assumption that our nets, in their passage, would capture every individual shrimp, and every fish of appropriate size that lay in their paths. We may well leave this discussion at this point, because no definite information is available as to what proportion of the more active fishes would be likely to dodge the trawls, or to what extent this potential loss would be counterbalanced by the opposing tendency for the lead-ropes to the otter boards to turn inwards, toward the mouth of the net, such other fishes as might be to either side of the latter.³

² Crediting the *Glyptocephalus* and *Hippoglossoides* with an average weight of 1 lb. each.

³ For discussion of the relative efficiency of trawls rigged in this way—the so-called "V.D." system—as compared with those with the boards close in to the net itself, see Hickling, 1931.

Summary

Findings from 22 trawl-hauls, at 20 stations on mud bottoms in the western and northern parts of the Gulf of Maine, in depths between 120 and 228 meters, August, 1936, are:

1. The faunal community of the water just above these deep mud bottoms is so monotonous that the catch consisted, in every case, almost wholly of five species of fish (*Sebastes marinus*, *Merluccius bilinearis*, *Urophycis tenuis*, *Hippoglossoides platessoides*, *Glyptocephalus cynoglossus*) and of the shrimp, *Pandalus borealis*, in varying combinations of relative abundance. The only exception was at one station where a larger catch was made of crabs (*Geryon*) than of shrimp.

2. The only other fishes taken were odd examples of the 19 species listed on page 309. The total number of species to be expected in such situations (except as accidental strays) is probably not more than 35.

3. As the nets were arranged to fish off bottom, benthonic invertebrates were not sampled.

4. The most significant faunal contrast, from the regional standpoint, is that *Pandalus* has a well-marked center of population, at least in summer, in the deep bowl west of Jeffrey's Ledge where bottom currents are weak. A positive correlation was also shown between the amounts of shrimps caught at different stations and the percentage of organic matter in the mud, corroborating Hjort and Ruud's (1938) earlier conclusion that *Pandalus* is abundant only where the supply of organic debris on the bottom is relatively large.

5. *Merluccius* was also most plentiful in this bowl, probably in pursuit of shrimp. But the catches do not support any prevailing contrast between this locality and the open basin of the Gulf, in the abundance of any one of the other four fishes of which large catches were made. Neither is any regional contrast suggested in the qualitative composition of the fish fauna over mud bottoms in different parts of the Gulf at the depths in question.

6. The stock of *Merluccius*, in the localities and depths studied, was strongly dominated by the yearlings, an age group the habitat of which in the Gulf of Maine was previously unknown, though the older fish are extremely plentiful there in shoaler water. But the catches of *Sebastes*, of *Hippoglossoides*, of *Glyptocephalus*, and of *Urophycis tenuis* consisted chiefly, or (in the last case) wholly of fish of two years and upwards. The few cod that have been taken in the bowl from time to time have all been very large, though smaller cod as well find their way down into the deeps of the open basin of the Gulf.

7. Length-frequency distribution of the catches added to previous information, indicates that *Sebastes*, in the Gulf of Maine, attains an average length of about 7.5 cm. at one year of age and of about 18 cm.

at two years. But subsequent growth is so slow that age-analysis of larger sizes would require a larger series of measurements than are yet available. Yearlings of *Merluccius* average about 17 cm. by late summer in the deep waters of the Gulf, the two-year-olds about 25 cm.

8. It is estimated that the combined weight of shrimp and fish of all species, in the bowl west of Jeffrey's Ledge, at depths greater than 150 meters, would have averaged at least 576 lb. per haul of 1.5 sea miles with an 82-foot trawl, or 55 lb. per acre of bottom, had the 6-inch stratum next the sea floor been included.

BIBLIOGRAPHY

- BIGELOW, HENRY B., 1927. Physical oceanography of the Gulf of Maine. *Bull. U. S. Bur. Fish.* **40** (Part II): 511-1027, 207 text figs.
- BIGELOW, HENRY B., AND WILLIAM C. SCHROEDER, 1936. Supplemental notes on fishes of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **48**: 319-343.
- BIGELOW, HENRY B., AND WILLIAM W. WELSH, 1925. Fishes of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40** (Part I): 1-567, 273 text figs.
- BIRDSEYE, CLARENCE, 1928. Shrimp Fishin Out o' Gloucester. The Story of a New Industry. *Fishing Gazette*, **45** (No. 4, April 1928): 12-13, 3 text figs.
- BROCH, HJALMAR, 1935. Die Bedeutung der Drøbak-Schwelle für die Bodenfauna der Garnelen-Felder. *Avhandl. Det Norske Vid.-Akad. Oslo*, I. Mat.-Nat. Kl. 1935, No. 5, pp. 1-32.
- GOODE, GEORGE BROWN AND TARLETON H. BEAN, 1896. Oceanic ichthyology. A treatise on the deep-sea and pelagic fishes of the world based chiefly upon the collections made by the steamers "Blake," "Albatross," and "Fish Hawk" in the northwestern Atlantic, with an atlas containing 417 figures. *Mem. M. C. Z.*, Vol. XXII, pp. I-XXXV, 1-553. Also, as *Smithsonian Contributions to Knowledge*, Vol. XXX, (1895) 1896, and as *Special Bulletin No. 2, U. S. Nat. Mus.*, 1895 (issued June, 1896).
- HICKLING, C. F., 1931. A comparison of the Vigneron-Dahl trawling gear with the steam otter trawl, in the hake fishery. *Cons. Int. Perm. Explor. de la Mer, Jour. du Conseil*, **6** (No. 3): 421-431.
- HJORT, JOHAN AND JOHAN T. RUUD, 1938. Deep-sea prawn fisheries and their problems. *Hvalrådets Skrifter, Norske Videnskaps-Akademi i Oslo*, No. 17, pp. 1-144, 21 text figs.
- MOLANDER, ARVID R., 1925. Observations on the witch (*Pleuronectes cynoglossus* L.) and its growth. *Cons. Int. Perm. Explor. de la Mer, Publ. de Circ.*, No. 85, pp. 1-15, 4 text figs.
- RATHBUN, RICHARD, 1883. Notes on the shrimp and prawn fisheries of the United States. *Bull. U. S. Fish Comm.*, **2** (for 1882): 139-152.
- RATHBUN, RICHARD, 1884. Part V. Crustaceans, Worms, Radiates, and Sponges. In Goode, George Brown, 1884, *The Fisheries and Fishery Industries of the United States. Section I, Natural History of Useful Aquatic Animals*, pp. 759-850.
- WAKSMAN, SELMAN A., 1933. On the distribution of organic matter in the sea-bottom and the chemical nature and origin of marine humus. *Soil Science*, **36** (No. 2): 125-147.
- WAKSMAN, SELMAN A., AND MARGARET HOTCHKISS, 1938. On the oxidation of organic matter in marine sediments by bacteria. *Jour. of Mar. Research*, **1** (No. 2): 101-118, text figs. 34-35.
- WALFORD, LIONEL A., 1936. Notes on shrimp fishing along the New England coast. *Memorandum, U. S. Bureau of Fisheries*, Ser. I, No. 57, 5 pp., 2 pls.
- WOLLEBAECK, ALF, 1903. Ræker og rækefiske. Aarsberetning vedk. *Norges Fiskerier*, 1903, vol. 2, pp. 167-229, 20 text figs.

TASTE THRESHOLDS IN LEPIDOPTEROUS LARVAE

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Introduction

The first attempt to measure the taste thresholds of lepidopterous larvae was made on eleven species including *Liparis dispar* L., *Danaus plexippus* L., *Papilio ajax* L., *Diacrisia virginica* Fabr., *Estigmene acrea* Dru., *Platysamia cecropia* L., and *Isia isabella* A. & S. (Dethier, 1937). The threshold concentrations were found to be approximately eight times less dilute than those of man. Eger (1937), working with several European species, obtained results almost identical for HCl but could get no definite reactions with sugar solutions.

The present paper represents an attempt to ascertain more accurately the threshold values for other substances in additional species. It also attempts to show that larvae of *Malacosoma disstria* Hbn. and *Apamea velata* Wlk. are sensitive to sugar solutions and that reasonably accurate threshold values can be determined.

Methods

Two species were tested, *Malacosoma disstria* Hbn. and *Apamea velata* Wlk. A few tests were also conducted with *M. americana* Fabr. In the case of *M. disstria* experiments were carried on in the field under natural conditions. Larvae were imprisoned upon the trunk of their food-tree between two encircling bands of Tanglefoot which prevented them from ascending or descending the trunk. In addition each animal was marked with a daub of paint. Thus the gastronomic history of each could be followed. Solutions to be tested were offered on the end of a fine glass rod. This method of experimenting with larvae in the field is most advantageous since such external factors as temperature and humidity are those of the natural environment of the animals. In addition, excessive handling is eliminated. Unfortunately such methods are not applicable to *A. velata* which lives close to the ground on low-lying herbage. In this case the experimental animal was allowed to crawl on a clean ground glass plate. A drop of the solution to be tested was then placed in the path of the larva. This general experimental procedure was also followed with a few specimens of *M. disstria* to

serve as a check against possible differences arising from the dissimilarity of the two procedures. Although it was shown that such differences did not arise, a few larvae living under laboratory conditions sickened and died while all those in the field eventually pupated.

All larvae were offered distilled water and those which accepted were eliminated from experiments with sugars. When testing with HCl only those animals were used which accepted water. Following this preliminary determination drops of the test solution were offered. Each test was immediately preceded and followed by a trial with distilled water which served as a control and as a means of cleansing the mouthparts. The order of presentation of solutions was constantly changed in order to exclude the possibility of association.

Thresholds are stated in terms of two concentrations indicating in the case of sugars that the animals refused the lower and accepted the higher; in the case of acids, that the animals accepted the lower and refused the higher. The lowest concentration detectable by the larva lies between these two values.

There were two possible sources of error, first that arising from diluting the stock solution, second that due to evaporation after the solutions had been made up. The latter source was eliminated to a large extent by making up fresh solutions directly preceding each series of experiments. The deviation from the calculated molarities in a series of sugar dilutions due to both of the above-mentioned sources combined was determined twice by measuring the specific gravity, first, directly upon the conclusion of a series of experiments. The deviation from the calculated molarities was ± 0.9 per cent. Second, a similar check was run on a series of solutions thirty days old. The determined values were now 15 per cent higher. This illustrates the need of working with fresh solutions. All the threshold values stated are the determined values. Sugar solutions were made from Merck and Mallinckrodt purest sugars with distilled water as a solvent. New unused glassware was employed and there was no evidence of bacterial contamination.

HCl

One hundred and thirty-eight tests were made with *M. disstria* and thirty with *A. velata*. Different molarities forming the following series were made up by careful dilution and offered to the larvae: .001M, .002M, .003M, .004M, .005M, .01M, .02M, .03M, .04M, .05M, .06M, .07M, .08M, .09M, .1M. The lowest threshold value obtained lay between .004M and .005M for both species. The lowest found by Eger lay between .003M and .006M. Dethier (1937) found a value between .0005M and .005M. The average threshold value for *M. disstria* was

.018M and .0085M for *A. velata*. Figure 1 illustrates clearly the difference in sensitivity to HCl between the two species. Though the lowest threshold value obtained was the same for both, it may be seen from the graphs that there was a greater percentage response to .005M HCl by *A. velata* than by *M. disstria*. A comparison of responses to .01M HCl reveals that the percentage response of *A. velata* at that concentration was just twice that of *M. disstria*.

Throughout the experiments *A. velata* larvae proved most difficult subjects. Occasionally there were feeble responses to .004M HCl but these were very doubtful. The average threshold was the same for

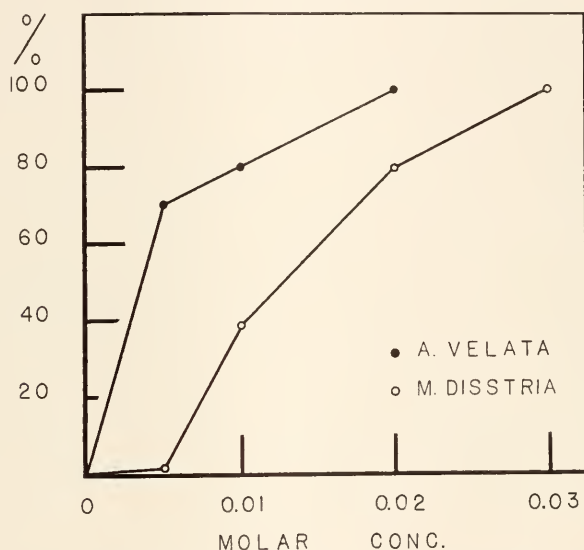


FIG. 1. Graphs showing the difference in threshold sensitivity of *A. velata* and *M. disstria* to HCl. The percentage response is plotted on the ordinate and the molar concentration of HCl on the abscissa.

first instar larvae as for last instar larvae of *M. disstria*. Indications are that a period of starvation lowers the acceptance threshold, but more work is necessary to confirm this.

Sucrose

Three hundred and nine tests were made with *M. disstria*; eighty tests, with *A. velata*. The following sucrose molarities were employed: .008M, .01M, .02M, .03M, .04M, .05M, .06M, .07M, .08M, .09M, .1M, .125M, .2M, .250M, .3M, .4M, .5M, .6M, .7M, .8M, .9M, 1M. The lowest threshold value obtained lay between .01M and .02M for *M. disstria*, between .01M and .02M for *A. velata*. The average threshold

value was .015M for *M. disstria*, .037M for *A. velata*. Again it was possible to detect no change in the average threshold value for larvae at different instars.

Fructose

Eighty-one tests were made with *M. disstria*; eighty-two, with *A. velata*. Tests were made with the following molarities: .008M, .01M, .02M, .03M, .06M, .0625M, .1M, .125M, .2M, .250M, .3M, .4M, .5M, 1M. For *M. disstria* the lowest threshold value obtained lay between .03M and .06M; for *A. velata*, between .01M and .02M. The average

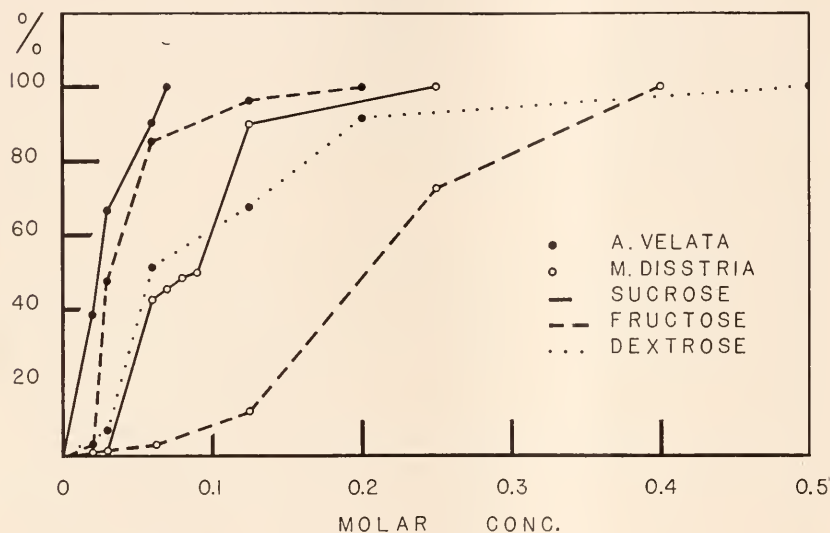


FIG. 2. Graphs showing the difference in threshold sensitivity of *A. velata* and *M. disstria* to various sugars and the order of effectiveness for these sugars. The percentage response is plotted on the ordinate and the molar concentration of the sugars on the abscissa.

threshold for the former was .273M, .058M for the latter. No age effect was demonstrable.

Dextrose

Sixty-eight tests were conducted with *A. velata*. Solutions from the following series were tested: .008M, .01M, .02M, .03M, .06M, .0625M, .1M, .125M, .2M, .250M, .5M, 1M. The lowest threshold value obtained lay between .02M and .03M. The average threshold value was .138M. *M. disstria* larvae failed to respond to dextrose even as concentrated as 1M.

Conclusions

On the whole, *A. velata* appeared more sensitive to taste substances than *M. disstria* (see Fig. 2). The order of sensitivity to the various sugars was the same for both species, namely, sucrose, fructose, dextrose, and lactose, in decreasing order of sensitivity. It was possible to demonstrate but a small response to maltose and none at all to lactose. *M. disstria* failed to respond to dextrose. *M. americana*, with three exceptions, failed to respond to any sugars. The three animals which did respond did so to .1M sucrose. Larvae which responded to sucrose concentrations as low as .02M refused lactose as concentrated as 1M; .1M sucrose was accepted and .1M lactose refused. Since lactose and sucrose of equal concentrations are supposed to have the same osmotic pressure and viscosity (Minnich, 1929), this indicates that responses were truly gustatory.

The previous dietary regime exercised a profound effect upon the threshold. Larvae which had been allowed to drink a quantity of sucrose always failed to respond to previously determined threshold concentrations of fructose or dextrose. Likewise an excess of fructose raised the acceptance threshold of dextrose. The reverse effect was not in evidence. This is taken as further evidence for the order of sensitivity to the various sugars mentioned above.

Although no age effect could be demonstrated (first instar larvae of *A. velata* had the same thresholds as last instar larvae), it was observed that twenty-four hours prior to pupation thresholds were exceedingly high. This condition seems to have been due to disinclination on the part of the larvae to eat rather than a rise in the threshold due to age.

Thus it may be seen that *M. disstria* and *A. velata* larvae react very definitely to sugar solutions. The values obtained for these two species compare favorably with those determined for other caterpillars.

LITERATURE CITED

- DETHIER, V. G., 1937. Gustation and olfaction in lepidopterous larvae. *Biol. Bull.*, 72 (1): 7-23.
- EGER, H., 1937. Über den Geschmacksinn von Schmetterlingsraupen. *Biol. Zentralbl.*, 57 (5/6): 293-308.
- MINNICH, D. E., 1929. The chemical sensitivity of the legs of the blow-fly, *Calliphora vomitoria* Linn., to various sugars. *Zeitschr. f. vergl. Physiol.*, 11 (1): 1-55.

BUDS INDUCED FROM IMPLANTS OF NERVE CORD AND NEIGHBORING TISSUES IN THE POLYCHAETE, *CLYMENELLA TORQUATA*

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The problem of the influence of the nervous system on the formation of new tissues has attracted the attention of a number of workers, with perhaps the majority of them interested in its effect on the replacement of lost parts. Extensive studies in this field have been carried out on annelid regeneration with considerable divergence of reported results. By some investigators (for example, Morgan, 1902, and Bailey, 1930) it is believed that the nerve cord is essential to regeneration. On the other hand, Goldfarb (1909), Siegmund (1928), and Avel (1932) were able to obtain regeneration in the absence of any ventral nerve cord in the immediate vicinity of the cut surface. Baskin (1933) believes that the nervous system exerts an essential but entirely unspecific influence on head formation in the earthworm. Crowell (1937) says, "The evocation of morphogenesis can be directly attributed to the nerve cord or brain. The nervous structures are thus comparable to organizers." He believes, however, that, "The region of the nerve cord concerned in regeneration does not determine the form of the regenerate." In a recent paper P. L. Bailey, Jr. and G. H. Bailey (1938) report concerning regenerating tissues that, "Organization of these tissues into normal new structures in *Eisenia* and *Nereis* seems to be dependent upon the presence of the nerve cord at the cut surface and in its normal relation to the other tissues."

In an attempt to learn more about this problem the experiments reported in this paper were undertaken, first in the summer of 1934 at the Marine Biological Laboratory, Woods Hole. Each operation consisted in removing a piece of tissue from one *Clymenella* and implanting it in the body wall of the thirteenth segment of another individual of the same species. Each implant was inserted in a dorso-lateral region near the longitudinal vessel which runs in the vicinity of the notopodial sac. Implantation in this region not only gives a plentiful blood supply but also removes any danger of injury, during the operation, to the nerve cord of the host.

In *Clymenella* the nerve cord lies outside of the circular muscles and is virtually a part of the integument (see fig. 14). In removing pieces of the cord, therefore, small amounts of ventral body wall about the cord were included. In all cases care was taken to keep this extra material at a minimum. One very important point to note, however, is that all pieces included some mesodermal elements which were very intimately associated with the nerve cord. It has been shown in a number of polychaetes that many neoblasts are present along the ventral nerve cord of an intact worm. Probst (1931) reported that neoblasts, present in this region of *Aricia*, produce practically all of the new tissues in posterior regeneration. Faulkner (1932) found a similar situation in *Chaetopterus*. Studies of *Clymenella* lead the author to believe that much the same condition also exists in this worm. It is clear then that the so-called nerve cord implants included at least a little hypodermis and some mesodermal elements including muscle tissue and probably many neoblasts.

For controls, pieces of body wall or wall of the digestive tract were used. The latter included, of course, both endodermal and mesodermal elements. The pieces of body wall probably included all of the general types of cells present in the nerve cord implants, with the exception of the neoblasts and the elements of the nerve cord itself.

Control Animals

Thirty worms served as hosts for control implants. Five of these implants were portions of the digestive tract, the others were taken from various parts of the body wall except in the vicinity of the nerve cord. Six of the hosts, including 3 with gut implants, died within a few days. Of the survivors, 13 lost the implants. The remaining 11, all with body wall implants, lived from 9 to 27 days. In none was there any evidence of bud formation. In all there seemed to be a certain amount of resorption of the implant. In those surviving 3 weeks or more a scar with possibly a minute pimple-like elevation was all which remained as external evidence of the operation.

Experimental Animals

A nerve cord implant was inserted in the dorso-lateral part of the thirteenth segment of each of 69 worms. Sixteen of these animals died within 10 days after the operation. In 30 other cases the implant either dropped out or slipped into the coelomic cavity. No buds formed on any of these.

Of the remaining 23 worms, in which the graft took, 6—including 2 which lived more than 30 days after the operation—showed no evidence

of new tissue at any time. Of the remaining 17, 3 produced irregular buds and 14 formed tails at the implant region. The development of these tail buds followed a quite consistent schedule. The first unquestionable evidence of new tissue was usually on the fourth or fifth day. By the seventh or eighth day a low anal collar with minute cirri had appeared, developing about the original dorsal side of the protruding portion of the implant in such a way that the external surface of the latter was exposed. Segmentation began to be evident on about the tenth day and was usually quite distinct, with notopodial setae well developed, two or three days later. The position of the notopodial setae indicated that the implant was in the mid-ventral part of the base of the bud.

Three of the tail buds consisted of ten segments, the proximal seven of which were setigerous. In the intact animal this is the number and organization of segments posterior to the implant point, if the thirteenth segment is included. Two of these buds (Figs. 1 and 2) did not differ appreciably in external appearance from the usual type produced in ordinary regeneration at this level. The implant came from the eleventh segment of the donor for the bud in Fig. 1 and from the second segment for the other bud. The third bud (Fig. 3) was curved toward the side in which the implant—taken from the second segment of the donor—was located. The anal collar was open on the same side. Evidently the amount of tissue produced along the ventral part of the bud was considerably less than in other parts. In ordinary posterior regeneration at the eighth to eleventh segments, buds with one side of the anal segment short or missing often occur (Sayles, 1934). Also, at the twelfth to fourteenth segments one side of the anal collar is usually shorter at first. In contrast with this implant bud, however, the open side is probably always dorsal in regenerates.

Four of the implant tail buds were of 9 segments. With the exception of a small part of the thirteenth segment, this is the number found in the intact worm posterior to the level of the implant. Two of these buds (Figs. 4 and 5) were of the ordinary tail type. The implants for these came from segments 10 and 8, respectively. Segment 8 also supplied the implant for another bud which was short, bent, and with a double anal segment (Fig. 6). The fourth bud of this group—implant from the sixth segment—terminated in an anal segment which was open on one side, only about one-half of the segment being present. From the last setigerous segment of this bud there arose a secondary part consisting of a slender stalk at the end of which was another anal segment, also open on one side. Unfortunately this worm died before a careful drawing had been made.

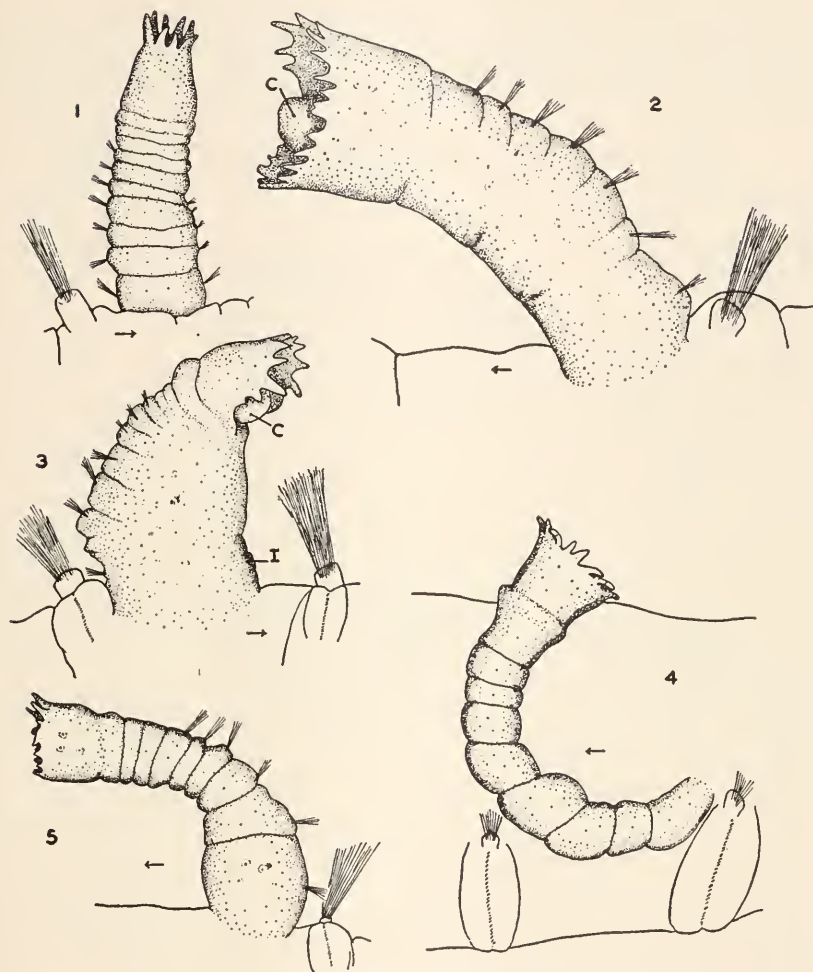


FIG. 1. Dorsal view of part of thirteenth segment of host bearing 10-segment induced tail bud. Age, 15 days. Arrow points toward anterior end of host in this and all other figures. Except where otherwise noted all figures are camera lucida drawings and $\times 22$.

FIG. 2. Dorsal view of part of thirteenth segment of host bearing 10-segment induced tail bud. Age, 15 days. *C*, anal cone.

FIG. 3. Ventral view of part of thirteenth segment of host bearing 10-segment induced tail bud. Age, 19 days. *C*, anal cone; *I*, exposed part of implant.

FIG. 4. Left lateral view of thirteenth segment of host bearing 9-segment induced tail bud. Age, 26 days.

FIG. 5. Ventral view of part of thirteenth segment of host bearing 9-segment induced tail bud. Age, 16 days.

One bud (Fig. 7) consisted of 7 segments, 4 of these setigerous, and terminated in an anal collar of the usual type. From the ventral side of the middle of this bud there had developed a three-segment secondary

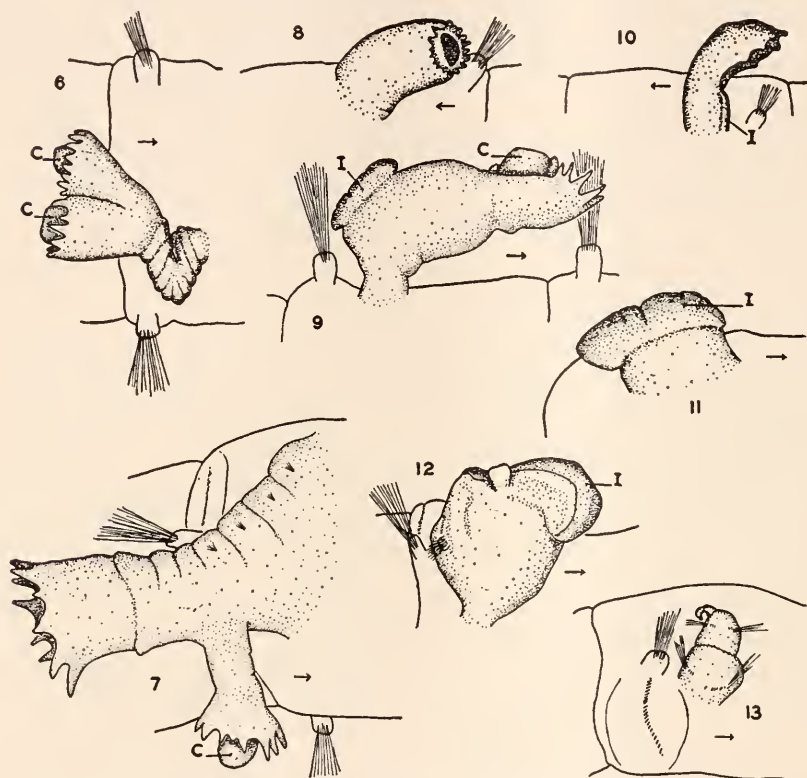


FIG. 6. Dorsal view of part of thirteenth segment of host bearing 9-segment induced tail bud with anal segment double. Age, 30 days. C, anal cone.

FIG. 7. Dorso-lateral view of part of thirteenth segment of host bearing induced double tail bud. Age, 18 days. C, anal cone.

FIG. 8. Dorsal view of part of thirteenth segment of host bearing induced tail bud. Age, 10 days.

FIG. 9. Ventral view of part of thirteenth segment of host bearing induced tail bud. Age, 14 days. C, lobulate anal cone region; I, exposed part of implant.

FIG. 10. Left lateral view of part of thirteenth segment of host bearing induced bud consisting almost entirely of anal segment open on one side. Age, 11 days. I, exposed part of implant. Enlarged from small, rough sketch. About $\times 20$.

FIG. 11. Right lateral view of part of thirteenth segment of host with implant, I, raised up on small amount of new tissue. Age, 15 days.

FIG. 12. Right dorso-lateral view of part of thirteenth segment of host with implant, I, raised up on mass of new tissue. Age, 44 days.

FIG. 13. Right lateral view of part of thirteenth segment of host bearing induced bud consisting of two setigerous segments followed by a slender, twisted region. Age, 26 days.

tail of good shape except for the large size of the anal cone. Microscopical preparations of this bud show a division of the nerve cord of the main bud running into the extra part. In this case the implant came from the tenth segment.

Three of the tail buds showed little or no external evidence of segmentation. This condition was probably due in part to the fact that they lived only 10–14 days after the operation. Two of these were of the usual type with complete anal collars but with exceptionally large anal openings. One of the latter (Fig. 8) was the result of an implant from the sixth segment; the other, lost before an accurate drawing was made, arose from an implant from the twelfth segment. The third bud in this group (Fig. 9) was bent sharply toward the anterior end of the host. The implant—from the seventh segment—could be seen at the outside of the bend. On the same side of the bud the anal segment was open.

Each of the three remaining tail buds consisted of little more than an anal segment open on one side. In two of these the exposed part of the implant was proximal to the open portion of the bud. No record was made of the position of the implant in the third. The third, fifth, and seventh segments supplied the implants for these. In each of these cases an endeavor was made to keep the animal alive to observe further developments. As a result not one was preserved or carefully drawn. Figure 10 was prepared from a rough sketch of one of these, made 11 days after the operation.

Of the three irregular buds, two apparently consisted of the implant raised upon a small mass of new tissue. This new tissue showed no evidence of differentiation in one case (Fig. 11) but included a single notopodium in the other (Fig. 12). The third irregular bud (Fig. 13) was made up of two well-formed setigerous segments followed by a slender, hook-like distal region. This condition was probably not the result of the breaking off of the tip of a developing bud as the bud was slender when it was first forming on the eighth day and the end began to curve dorsally on the tenth day. The implants in these cases came from the eleventh, sixth and second segments, respectively.

Some of the internal features associated with these buds are well illustrated by the bud of Fig. 8. An oblique section through the base of this bud, showing its relation to both host and implant, is shown in Fig. 14. The implant forms a large mass lying in the coelomic cavity ventral to the digestive tract. The great thickness of the implant hypodermis, as contrasted with that of the host, is due in part to the fact that the implant is cut in an oblique plane but chiefly to the fact that it really was thicker. The body wall in the posterior part of *Clymenella* is thin,

somewhat transparent, and includes few glands. In the anterior region, on the other hand, there is a very thick, opaque and highly glandular integument. At the ninth segment there is a distinct line of demarcation between these two regions. This particular implant bud came from the anterior region (sixth segment).

Several points brought out by a study of serial sections of this animal apparently hold true of such other worms of this series as have been studied. The digestive tract of the bud is completely independent of that of the host. It is apparently formed by cells from the regeneration

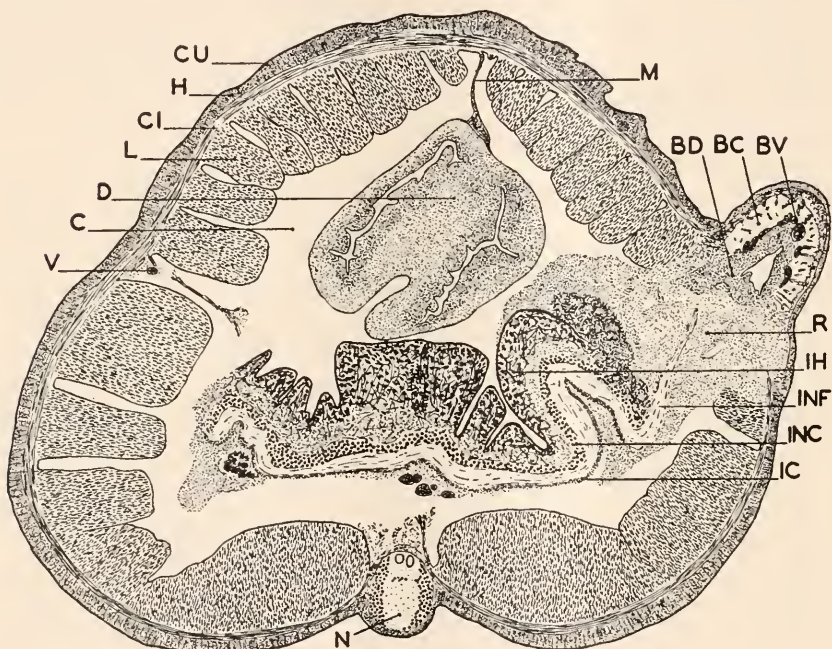


FIG. 14. Cross-section through host and oblique section through base of implant bud of Fig. 8. Age, 10 days. *BC*, coelom of bud; *BD*, digestive tract of bud; *BV*, blood vessels of bud; *C*, coelom; *CI*, circular muscle layer; *CU*, cuticle; *D*, digestive tract; *H*, hypodermis; *IC*, circular muscle tissue and regeneration cells of implant; *IH*, hypodermis of implant; *INC*, cell bodies of implant nerve cord; *INF*, fibers of implant nerve cord; *L*, longitudinal muscle; *M*, dorsal mesentery; *N*, nerve cord of host; *R*, mass of regeneration cells; *V*, lateral longitudinal blood vessel. $\times 40$.

mass. There is also no evidence of any continuity between nerve cords of implant and host. The tip of the implant cord, attached to the right side of the body wall, is directed toward the ventral side of the bud. The ventral side of both bud and implant is toward the dorsal side of the host. Thus the bud is developing at the end of the implant with

the same orientation as one would have found in a bud which might have regenerated at the end of this same nerve cord if the latter had been left in place in the donor.

Discussion

The buds described above have apparently all been produced as the result of the implantation of a piece of appropriate tissue from an adult, non-regenerating animal. At the present time studies are being carried on in an endeavor to learn: (1) what cellular elements are contributed by host and by implant; (2) whether the elements of the implant important in inducing the bud are the neoblasts or the nerve cord or both. Some evidence has already been obtained to indicate that the neoblasts may be essential. This appearance of a bud in association with an implant cord is not in accordance with the ideas expressed by P. L. and G. H. Bailey (1938) who found that in *Eisenia* and *Nereis* organization of tissues into normal structures is dependent upon the presence of the nerve cord "in its normal relation to the other tissues."

Of the 17 cases in which new tissue was formed at the implant, 14 were tail buds and 3 were irregular. In no case was a bud formed which in any way resembled a head, even of the modified type found in anterior regeneration at certain levels (Sayles, 1936). From the point of view of regeneration, the 22-segment body of *Clymenella* may be divided into three regions, namely: (1) segments 1-7, levels at which regeneration occurs in the anterior direction only; (2) segments 8-11, a transition zone in which lost posterior ends are more and more accurately replaced the more posterior the level and in which the reverse is true of lost anterior ends; (3) segments 12-22, levels at which anterior regeneration does not occur but lost posterior portions are completely replaced. Of the 14 implants associated with induced tail buds, 8 were from the anterior region of the donor, 5 were from the middle region, and 1 was from segment 12. (For the irregular buds 2 implants were from the anterior zone and one from the middle one.) Since the implant was put into the thirteenth segment of the host in each case, all were in the posterior, tail-forming zone. These data point clearly to the fact that at the thirteenth segment, the host and not the implant determines the nature of the bud to be formed. In 7 cases the great influence of the host is further indicated by the presence of buds of nine or ten segments, while nine and a fraction segments exist posterior to the implant. This is all the more impressive when we note that two of the 10-segment buds resulted from implants taken from the second segment, a level at which only a single head segment may ordinarily regenerate. These results are in accord with those of Baskin (1933) and Crowell (1937).

The implant does, however, seem to control the orientation of the new bud. In each bud which developed at the exposed end of an implant the dorsal and ventral parts bore the same relation to the implant as one would expect to find between nerve cord and bud in ordinary regeneration. For example, in the case of the bud shown in Fig. 3 the implant was placed with its ventral surface toward the anterior end of the host. When the bud developed its ventral side was toward the anterior end of the host and the implant showed in the proximal part of this side.

A tentative explanation may be offered for the condition found in two of the irregular buds (Figs. 11 and 12). In each of these cases the implant was relatively short and healed into the wound region throughout most of its length. As in other cases the new tissue developed on the original internal, or dorsal, side of the implant. In these two worms this new tissue was, therefore, beneath the implants and raised them up from the body wall of the host. Under these conditions the new tissue formed was apparently very limited in amount and in differentiation.

LITERATURE CITED

- AVEL, M., 1932. Sur une expérience permettant d'obtenir la régénération de la tête en l'absence certaine de la chaîne nerveuse ventrale ancienne chez les Lombriciens. *Compt. Rend. Acad. Sci. Paris*, **194**: 2166-2168.
- BAILEY, P. L., JR., 1930. The influence of the nervous system in the regeneration of *Eisenia foetida* Savigny. *Jour. Exper. Zool.*, **57**: 473-509.
- BAILEY, P. L., JR. AND G. H. BAILEY, 1938. Further experiments on the influence of the nerve cord in posterior regeneration in *Eisenia* and *Nereis*. *Jour. Exper. Zool.*, **79**: 13-33.
- BASKIN, B., 1933. Über den Einfluss des Nervensystems auf den Regenerationsvorgang beim Regenwurm. *Zool. Anzeig.*, **103**: 253-262.
- CROWELL, P. S., JR., 1937. Factors affecting regeneration in the earthworm. *Jour. Exper. Zool.*, **76**: 1-33.
- FAULKNER, G. H., 1932. The histology of posterior regeneration in the polychaete *Chaetopterus variopedatus*. *Jour. Morph.*, **53**: 23.
- GOLDFARB, A. J., 1909. The influence of the nervous system in regeneration. *Jour. Exper. Zool.*, **7**: 643-722.
- MORGAN, T. H., 1902. Experimental studies of the internal factors of regeneration in the earthworm. *Arch. f. Entw.-mech.*, **14**: 562-591.
- PROBST, G., 1931. Beiträge zur Regeneration der Anneliden. I. Die Herkunft des Regenerationsmaterials bei der Regeneration des kaudalen Körperendes von *Aricia foetida* Claparède. *Arch. f. Entw.-mech.*, **124**: 369-403.
- SAYLES, L. P., 1934. Regeneration in the polychaete *Clymenella torquata*. II. Effect of level of cut on type of new structures in posterior regeneration. *Physiol. Zool.*, **7**: 1-16.
- SAYLES, L. P., 1936. Regeneration in the polychaete *Clymenella torquata*. III. Effect of level of cut on type of new structures in anterior regeneration. *Biol. Bull.*, **70**: 441-459.
- SIEGMUND, G., 1928. Die Bedeutung des Nervensystems bei der Regeneration, untersucht an *Eisenia*. *Biol. Generalis*, **4**: 337-350.



THE EFFECTS OF CENTRIFUGING ON THE POLAR SPINDLES OF THE EGG OF CHAETOPTERUS AND CUMINGIA

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The factors involved in the movement of the maturation spindle to the pole of the egg are obscure and have been little commented on. In many eggs it has been shown that the mitotic rays may extend through almost the entire polar hemisphere. In such eggs the axis of the spindle is described as at first horizontal, or possibly somewhat oblique. It then turns with one end directed to the pole, but whether there is one particular end that takes this position or whether either end may so orient is unknown. It is conceivable, if there is a predetermined end, that it may trace back to the last oogonial division, or if not, that a cytoplasmic difference may determine the direction of its turning. At present there is no evidence for either of these assumptions.

In some respects the eggs of *Chaetopterus* offer excellent material for the study of the conditions that lead to the movement of the meiotic spindle to the pole. The eggs can be procured in any quantity, and the same individual will supply material for two or more weeks. When removed from the parapodia the germinal vesicle is present in the eggs. It breaks down within a few minutes and the polar spindle forms, which moves within ten minutes to the pole of the egg where it remains until the egg is fertilized. The first polar body is given off 20 to 22 minutes after the egg is fertilized, and the second polar body about ten minutes later. Since all the eggs are in the same stage an opportunity is given to study the effects of centrifuging on the spindle at any stage desired.

If the egg is centrifuged at the time when the wall of the germinal vesicle is disappearing, and centrifuging is continued during the time when the polar spindle is being formed deep in the egg, the spindle may be prevented from passing to the pole or to any other point on the surface. Whether it does or not may depend in part on the centrifugal force employed; in part on the orientation of the egg during centrifuging; in part on the destruction of the peripheral rays of the mitotic figure; and in part on the redistribution of the contents of the egg.

If the egg is centrifuged after the polar spindle has reached the pole

its position there or its change of position will depend on the orientation of the egg with respect to the centrifugal force. If the egg is centrifuged during the time of extrusion of the polar body the result may again depend on its position with respect to the centrifugal force and, theoretically, at least, on whether the spindle is in anaphase or telophase or whether the second polar spindle is in process of formation.

Again, if the egg is centrifuged after the first polar body has been given off, the second spindle may remain in place or be driven into the interior. If the latter occurs the question arises whether it will return to the surface and give off the second polar body, or remain in the interior of the egg. These alternatives may depend on the location of the pole and also on whether the egg is elongated by the centrifugal force, or remains spherical or nearly so.

The first problem then is to find out whether the eggs do or do not orient on the machine with the polar hemisphere turned toward the center of rotation. In *Chaetopterus* the orientation of the eggs on the machine has been discussed by Whitaker and Morgan (1930), Wilson (1930), and Morgan (1937b, 1938). It has been found that there is a tendency for most of the eggs to orient more or less with the polar hemisphere toward the center of rotation, but in a considerable number of cases the pole may make any angle with the centrifugal axis up to as much as ninety degrees or even more. I have suggested (1938) that the failure of many eggs to orient perfectly may be due to the contents of the eggs beginning to respond to the centrifugal force more quickly than the egg as a whole. The best evidence for this is found when the eggs are centrifuged after the first polar body has been extruded. Under these circumstances the polar body frequently does not lie at the centripetal pole, but to one side of it. In such cases there can be no question of a change in the position of the true pole. If the eggs are strongly centrifuged before the spindle has reached the pole there is the possibility that the presence of the oil cap may interfere with the extrusion of the polar body in the oil field. This possibility has also been examined. If the centrifugal force is not too strong the oil field extends as a broad thin cap over the centripetal pole, and the spindle may pass through its center, or in most of the eggs at one side. In other eggs the spindle lies outside the cap. On the other hand, if the centrifuging is strong, or over a longer time, then the oil cap is smaller in circumference and extends deeper into the egg. If, as seems to be the case, fewer polar bodies are given off in its center, this is in part due to the small arc it covers, but possibly the oil may then interfere with the passage of the spindle through it. In such cases the spindle may pass to the surface outside the oil cap, which may mean that it does not come

off exactly at the true pole but near it. There is no serious objection to such an assumption, but it is difficult to prove because other evidence shows that the egg fails in many cases to orient with the pole exactly in the axis of rotation.

The way in which the stratification of the materials of the egg takes place has been little discussed by those who have used the centrifuge to separate the materials of the egg. If it is assumed that at first the oil, the clear cytoplasm, the yolk and the pigment are equally distributed in the egg, they may be considered as passing by or through each other according to their specific weights. On the other hand, these differences may be of such a kind that one of them, the oil for instance, may move more quickly to one end than does the yolk or the pigment to the opposite end, while the clear protoplasm comes to occupy the middle zone. Determinations of the so-called absolute viscosity of the egg, by measurements of the time it takes to bring about a visible stratification of these materials, are open to the criticism that some of the materials reach their positions sooner than do others. For example, the yolk and the pigment of the egg of *Cumingia* may take a longer time to be separated clearly than does the oil and its underlying clear zone. If the clear zone is the matrix from which the other materials are separated, then, I suppose, it is this matrix that is responsible for the viscosity, but I am not sure that this is the correct interpretation. However, in most cases reported, comparisons are made between eggs in different stages of maturation, and such comparisons may be more nearly valid.

When the ripe ovarian eggs, or eggs just set free, are killed in Flemming and later sectioned and stained there is found a ring of black granules in the protoplasm around the nucleus. The nucleoplasm remains unstained in iron haematoxylin. After the germinal vesicle breaks down this region stains blue. If the ovarian eggs are killed in aceto-carmine, sectioned, and stained in iron haematoxylin fewer dark granules are seen. Whether there are two kinds of granules, or, in the latter case, fewer are stained is uncertain.

If the egg is on the centrifuge when the germinal vesicle breaks down the larger granules move along the periphery towards the centrifugal end forming there a semicircle in section (Fig. 39). They are imbedded in a somewhat homogeneous layer that stains yellow in eosin which is thickest around the antipole outside the granules. In the center of the outer hemisphere there is a clear spherical area, sometimes having a flocculent appearance slightly stained by eosin. In it are a few dark granules. This clearer sphere does not appear to be due to failure of the preservatives to penetrate into it. Its origin is obscure. It looks as though it were the clear nucleoplasm, but that part of the egg is now

occupied by a finely granular stained zone (as it is also in the uncentrifuged egg). Over this clear sphere there is a crescent of very finely granular substance that stains very dark in iron haematoxylin (Fig. 40). The chromosomes are sometimes imbedded in this crescent or lie just outside it.

The eggs of some other species of animals have a more protoplasmic layer around and beneath the pole, while the yolk lies mainly in the antipolar hemisphere. If such eggs are centrifuged, and orient on the machine, the oil present in the clear region or in the yolk must pass through the protoplasmic region while the yolk simply becomes more condensed in the opposite hemisphere, leaving the clear zone nearly in its original place. But if the egg is not so oriented at the time when the redistribution begins there is an alternative possibility. The protoplasmic layer may move around one side of the egg, and the yolk around the opposite side. The polar spindle, if not attached to the surface, may then be carried along with the protoplasmic layer. When removed from the centrifuge, the stretched protoplasm may move partly back to the original polar field, and the polar body be given off at or near the pole. I have shown this to be the case in the egg of *Ilyanassa*, and there is some evidence that such a slipping around may take place in the centrifuged egg of the frog, even in response to gravity in an inverted egg. In *Crepidula* a similar movement may take place although Conklin's figures do not give any evidence of such a rotation.

In *Ilyanassa* the rotation of the interior of the egg was best demonstrated either by holding the egg in an inverted position in a small tube or by imbedding inverted eggs in gelatin (dissolved in sea water) that was solidified before placing on the machine (Morgan, 1935). In the latter experiment the question may arise whether the gelatin itself affects the result. The same question has arisen in the course of the present experiments when eggs were centrifuged on top of a gum arabic solution, or in or above an "isotonic solution" of cane sugar or raffinose. In fact, these methods were abandoned after the first experiments because it was found that some of the solutions not being quite isotonic caused the egg to shrink slightly and possibly inhibited, for a time, the movement of the spindle, or interfered with its movements on the centrifuge. Even when the eggs come to lie on top of a gelatin or gum arabic solution they lie in a gradient where the sea water mixes with the gum arabic, etc. This effect will be considered later. It suffices here to say that the stratification of the eggs can be as perfectly brought about when they lie on the gum as in sea water alone, but in most cases these eggs do not elongate or break apart as in sea water, and if kept too long in the solution the extrusion of the first or second polar body may be suppressed.

*Centrifuging the Egg during the Dissolution of
the Nuclear Membrane*

By means of the centrifuge it is possible to prevent the polar spindle from moving to the pole; or later, after it has moved to the pole, to carry it back into the interior of the egg. Under these conditions there are two alternatives. The chromosomes may then separate, but whether the mitotic figure will be capable of dividing the egg into equal or unequal cells, or whether the double or quadruple set of chromosomes remains in place and takes part later in the cleavage division can only be determined in each kind of egg by examining it. The other possibility is that during the first or the second polar division one end of the spindle, attached to the surface, may remain in place and the other end be carried deeper into the egg (as Conklin has described for the egg of *Crepidula*), and bring about a division sometimes into two large cells. In *Chaetopterus*, and also in *Cumingia*, I have not succeeded in doing this—the spindle and its attached chromosome either remaining at the surface after centrifuging, or moving as a whole deeper into the egg. Different eggs probably behave differently in this respect. In *Ilyanassa* the polar spindle in “inverted eggs” is carried along in the protoplasm at or near the surface of the egg while in *Crepidula* the spindle is driven through the center of the egg or at least it appears to do so judging by Conklin’s drawings, although he does not specifically state this to be the case. This kind of result may depend to what extent the eggs orient on the machine; for, if some or many of them lie obliquely, when centrifuging begins it is to be expected that the lighter and heavier parts may slip around each other rather than interpenetrate.

Both in *Ilyanassa* and in *Crepidula* a spermatozoön has entered the egg while in the oviduct, hence the polar spindle is activated. On the other hand, *Chaetopterus* does not give off its polar bodies unless the egg is fertilized. Such eggs must be fertilized in order to activate the mitotic division. The egg can be activated by the sperm either before it is centrifuged, when the conditions are more nearly comparable to the eggs of the other animals, or it can be first centrifuged and then fertilized. Both tests have been made. It is also possible to break apart the eggs on the centrifuge and obtain large or small tops. It might seem that the likelihood of division by means of the polar spindle would be thus facilitated, but this is found not to be the case. Even very small tops may give off small polar bodies.

When many eggs are centrifuged in sea water they make a compact mass at the bottom. In squirting them loose, with a long large-mouthed pipette, some will, no doubt, be broken apart if they have become elongated and have a narrow neck, but it is surprising how many even

of the latter remain intact. Unless the membrane is broken the eggs do not greatly elongate, but if the centrifugal force is strong enough the membrane breaks at the yolk region and the yolk squeezes out leaving the rest of the egg surrounded by the shriveled membrane. Sometimes more, sometimes less, of the egg escapes, and it may be pinched apart at almost any level. The oil, too, after heavy centrifuging, may pinch off, and either float freely or remain inside the membrane.

In order to avoid breaking the eggs apart and still elongate them for an examination of the subsequent behavior of the polar spindle, they were sometimes centrifuged over a sugar solution heavier than sea water or over gum arabic. This method avoids packing the eggs together at the end of the tube as they do in sea water. The packing can be avoided by adding only enough eggs over the sugar or gum to form a single layer on the surface. It was found, however, that the sugar, and the gum also that was used, bring about some shrinkage of the egg and that the movements of the spindle may be retarded or even stopped. This method had, therefore, to be abandoned since in many of the experiments it was desired to move the spindle and not interfere with its activity, for, if it is held back, along with the rest of the egg, it will, when the eggs are returned to sea water (where they quickly become more or less spherical), proceed under most conditions either to come to the surface and produce polar bodies or else skip that stage if held as long as an hour on the centrifuge, hence defeating the purpose of the experiment.

Formation of Polar Spindle

Since the condition of the egg at the time of centrifuging had a bearing on the results, it became necessary to prepare a normal series of stages for comparison. When the egg of *Chaetopterus* is removed a large nucleus is present in the center of the egg that has an irregular contour. In the living egg its outlines disappear in a few minutes and in the course of 11 minutes a clear oblong area appears at the pole. Stained preparations show that the chromosomes have not yet reached the pole at the time when the clear area is first formed. They lie near the center in a cluster or clump for 9 minutes. Nine tetrad chromosomes are present, one smaller than the others. At 12 minutes they lie in a spindle that is moving to the surface.

Centrifuging the Eggs in Sea Water

Several different experiments were made in which the time of centrifuging and the force applied to eggs in different stages of polar body formation were tested in order to find out whether the polar spindle, if

driven into the egg, would cause the egg or an egg-fragment to divide by means of the polar spindle, or return to the surface.

In some of the experiments the unfertilized eggs were centrifuged at the time when the chromosomes had just emerged from the germinal vesicle. The chromosomes are then near the center of the egg in the polar hemisphere and it might be supposed that they could be held there during the time that it takes for the spindle to form and move to the pole. This is in fact what sometimes happens. If then the eggs are removed from the centrifuge and fertilized an opportunity might seem to be given for the buried spindle to divide the egg, but in no case did this happen. The chromosomes either remained in the interior and took part in the later division of the egg, or, under some conditions, moved to the surface and gave off one (usually) or two polar bodies before the cleavage came on.

The alternative, viz., to fertilize the eggs at the time when the polar spindle has formed and then centrifuge them, was also tried. Under these conditions it seemed possible that the activated spindle might divide the egg while on the centrifuge, or later, if taken off at the proper intervals; but again the results were negative. The spindle moved to the surface and gave off one or two polar bodies, or remained in the egg and the chromosomes took part later in the cleavage.

Several attempts were made to drive the polar spindle (after fertilization) into the interior at the time when it was about to divide to produce the first polar body but, as a rule, the spindle and its attached chromosomes either remained at or near the surface, or if driven into the interior, it failed to divide the egg at the time of polar body formation. Since, in previous experiments with *Ilyanassa*, it had been found, though very rarely, that the second polar spindle may divide the egg (if it is elongated at that time), a number of experiments were made with this stage but without success.

In nearly all cases stained preparations were made of the eggs in order to check up the records of the living eggs. These confirmed the observations on the living eggs, but added very little further information except with respect to the location of the chromosomes.

It is obvious, then, if the eggs fall, to some extent, at random on the centrifuge, that the spindle (and chromosomes) would have to move across the diameter of the egg or through its center or along one side in all cases where the pole of the egg happened to be at the centrifugal pole, since the chromosomes come to lie in the protoplasmic zone of the centrifuged egg, or near it, or in the oil field. On the other hand, in the reverse situation the spindle (and chromosomes) would, if it moved at all, shift to the interior. In all intermediate positions the chromo-

somes would already lie at or near the level toward which their specific gravity tends to locate them. It has been shown in previous papers that the egg of *Chaetopterus* on the centrifuge tends to orient on the machine with the true pole more or less turned towards the center of rotation. Hence, in the majority of cases, there would be little tendency to shift the level of the spindle, that had already reached the surface, to a new position. In fact, this corresponds with the observations on preserved eggs centrifuged at that time. Practically no spindles were found at the apex of the oil but not infrequently at its border. Also none were ever found in the yolk that occupies about half of the egg. If a spindle happened to be in what becomes the yolk hemisphere after centrifuging (in an egg completely inverted), it would be driven into the lighter half of the egg.

Though it has not been possible to induce the polar spindles to act as a division center for the whole egg, or even for a part of an egg, the result is not due to a destructive action on the spindle, since it may after centrifuging move to the surface and give off one or two polar bodies. The failure is also not due to the egg not being elongated at the time when the spindle division is due, since very many cases of elongated eggs were observed. It may be said then that the failure of the polar spindle to divide the egg of *Chaetopterus* is due in part to the tenacity with which the spindle adheres to the surface after it has once reached the surface (except as explained above), or, in other cases, to the strong tendency for the spindle to come to the surface. But granted that these conditions occur in most of the cases, it still remains unexplained why the polar spindle, if held inside the egg during the time of its normal formation, is unable to divide the egg, or a fragment of the egg. In this connection, it should be recalled that the division of the egg of *Crepidula* or of *Ilyanassa* to form the so-called giant polar body is a relatively rare event.

Details of Centrifuging

(1) The eggs were fertilized 2 minutes after removal, and 5 minutes later were centrifuged at 2080 r.p.m. at 19½ cm. from center for one-half hour. When removed (Figs. 1, 2) a group of chromosomes was present either in the center of the stained zone or at one side. The eggs were not much elongated. No polar bodies had yet been given off. Later the eggs rounded up and a few showed polar bodies. One hour and 5 minutes after fertilizing about half of the eggs had divided. *Comment:* The polar spindle had not reached the surface (after 7 minutes) when the eggs were centrifuged. They were kept on the machine for a half hour during which time the first polar body was overdue. The stained preparations show that the chromosomes had come to the surface on the machine, but the polar body was not extruded. It is probable that the formation of the polar spindle had been interfered with. Even after removal few polar bodies were found.

(2) Three minutes after removal the eggs were fertilized and kept for 13 minutes. They were then centrifuged for 5 minutes at 1110 r.p.m.; for 5 minutes

at 2080 r.p.m.; and for 5 minutes at 2750 r.p.m. The eggs were somewhat elongated (Fig. 3). No polar bodies had been given off. A group of chromosomes surrounded by a clear area was present in the stained zone. The center of the yolk was more translucent than the periphery. Some of the eggs gave off one or two polar bodies 20 minutes after the normal eggs. Later many eggs divided, most with one, a few with two polar bodies; but in some eggs no polar bodies were found. *Comment:* The polar spindle must have reached the surface (after 16 minutes) before the eggs were centrifuged. After 15 minutes on the centrifuge no polar bodies had been given off although overdue about 10 minutes. Later some eggs showed a polar body 10 minutes after those of the normal control. It appears that the polar spindle had retreated or had been driven into the interior, but later, in some of them at least, it came to the surface and gave off one or two polar bodies when returned to sea water.

(3) Five minutes after removal eggs were fertilized and 11½ minutes later were centrifuged at 2080 r.p.m. for 5 minutes; then for 5 minutes at 2750 r.p.m. When removed the eggs were somewhat elongated (Fig. 4). The chromosomes are at the surface of the stained zone. A spindle is not evident. The centrifuging (after 16½ minutes) was too late or not strong enough to dislodge the chromosomes from the surface. The eggs showed polar bodies (one, or two?) 12 minutes after removal. The eggs had become more rounded. Antipolar lobes came in soon after this (Figs. 5, 6, 7). *Comment:* These eggs, in preparations, showed usually only one polar body, or none at all. The lobes in most eggs were in the yolk region, but it is noticeable that in many eggs the antipolar lobe came partly in the stained zone (Figs. 5, 6, 7), which means that such eggs had not oriented on the machine. Half of the eggs (those in the opposite tube) were kept on the centrifuge (at 2750 r.p.m.) for 7 minutes longer. They are more elongated, and the chromosomes are still attached to the surface (Fig. 8). Eggs of this lot were preserved 17 minutes after removal when some of them were dividing. The dividing eggs have one polar body; those not yet divided showed none in nearly all cases, although the large asters for the first division were present. *Comment:* The eggs were centrifuged about 4 minutes before the first polar body was due. They were on the machine for only 10 minutes. Polar bodies came off 10 minutes after removal, generally only one. Eggs of the same lot were centrifuged for 7 minutes more. They were more elongated and the chromosomes were at the surface. One polar body came off, but whether this corresponds to the first or the second normal polar body can not be stated. In either case the number of chromosomes after fertilization should be three-fold.

(4) After standing for 5 minutes the eggs were fertilized and then, after standing for 10 minutes more, were centrifuged at 1700 r.p.m. for 30 minutes. The eggs were somewhat elongated (Figs. 9, 10), but no polar bodies had been given off, at least from most eggs. A clear nucleus was found in some eggs (Fig. 9) and a bunch of chromosomes in others (Fig. 10). A few began to divide 5 minutes later. In some eggs, where the constriction for the first furrow is appearing at the top of the stained area, the oil is pinched off as a separate globule (Figs. 11, 12, 13, 14), and if it happens to contain the polar body nucleus *on the surface* it resembles a large polar body. But there can be no doubt that this is an event taking place long after the polar division took place. The eggs in the opposite tube were centrifuged for 8 minutes longer (Figs. 15, 16). They were more elongated; some gave off a polar body in or near the oil, others did not. A nuclear vesicle, or a group of chromosomes, lies in the stained zone. *Comment:* These eggs were centrifuged about 5 minutes before the polar body was due and kept on the machine for 30 minutes longer. No polar bodies were present, at least in most eggs, and a nucleus or a collection of chromosomes was present in the interior. Evidently the chromosomes had retreated from the pole, but the polar spindle did not divide the egg while on the machine.

(5) After 7 minutes in sea water the eggs were fertilized, and stood for 10

minutes in sea water when they were centrifuged at 1110 r.p.m. for 40 minutes. When removed the eggs were elongated and, some at least, had given off a polar body (or two) (Figs. 17, 18). They began to divide 10 minutes after removal (Fig. 19). *Comment:* These eggs were centrifuged about 3 minutes before the polar body was due. Some eggs gave off a polar body while on the machine. They divided 10 minutes after removal (Fig. 20), without polar bodies or sometimes with only one.

(6) Eggs, after standing 5 minutes, were fertilized and stood for 10 minutes. They were centrifuged at 2080 r.p.m. for 25 minutes and for 15 minutes at 2750 r.p.m. They were much drawn out and broken; many middles were present. Eggs in one of the tubes were centrifuged for 12 minutes more. These too were much drawn out and many broken. The cylindrical eggs contracted and 12 minutes later some of the whole eggs showed the oil pinched off (Fig. 21). Some of the middle also showed a constriction (Fig. 22). This was 10 minutes at least before the cleavage was due. Without an examination of stained preparations the large clear globules containing oil might seem to be giant polar bodies, but preparations show they are without nuclei and quite clearly due to partial fusion of top and middle. *Comment:* The eggs were centrifuged 5 minutes before the polar body was due, and at a higher speed than the preceding for 40 minutes. One or two polar bodies had been given off, and after removal a few eggs showed the oil region constricted off, but since these bodies did not contain a nucleus and often had one or two polar bodies at the surface, it is clear that these bodies are due to the protoplasm pinching in two, and not due to a division by the polar spindle.

(7) Eggs were kept in sea water for 3 minutes, then fertilized and after 5 minutes were centrifuged at 1110 r.p.m. for 3 minutes; at 2080 r.p.m. for 2 minutes; and at 2080 r.p.m. for 15 minutes. The eggs were elongated (Figs. 23, 24). The chromosomes were at the surface in the stained area, generally in the more faintly stained neck, sometimes in two groups. Rarely a polar body was found, though, as a rule, no polar bodies had been given off. The eggs were contracted 8 minutes afterwards (Figs. 25, 26), but no polar bodies were present. Later some of the eggs divided without polar bodies; other eggs had not divided at this time. *Comment:* The eggs were centrifuged 8 minutes after removal, hence about 12 minutes before the polar body was due and probably before the spindle had reached the surface. They were on the centrifuge for 20 minutes. The polar body was overdue when removed. The preparations show the polar chromosomes at the surface but only rarely a polar body had been given off.

(8) Eggs stood 5 minutes in sea water and were then fertilized and after 8 minutes more were centrifuged at 2080 r.p.m. for 10 minutes. The eggs were oval. Preparations show a group of chromosomes in the center of the egg in the stained zone. No polar bodies were observed in the living egg, or in the preparations. Most of these eggs cleaved at the same time as the normal. In the opposite tube the rest of the eggs were centrifuged at 2750 r.p.m. for 10 minutes longer. These were more elongated. The chromosomes were still in the center of the stained zone (rarely at the surface). No polar bodies came off, and no giant polar divisions were observed. *Comment:* The eggs were centrifuged 13 minutes before the polar body was due. The spindle must have been at or near the surface when the eggs were put into the machine. Centrifuged for only 10 minutes the eggs were not much elongated. The chromosomes were found in the preparations in the interior of the stained zone. No polar bodies came off after removal and the eggs cleaved at the normal time. Other eggs of the same lot, centrifuged 10 minutes longer, gave the same results.

(9) The next set was not fertilized. After standing 6 minutes the eggs were centrifuged for 10 minutes at 1110 r.p.m.; then for 10 minutes at 2750 r.p.m. (Figs. 27, 28). The eggs were, for the most part, separated into middle and bottom; the oil was off in most cases. In the middles the chromosomes were found at the surface (in clumps) (Figs. 27, 28). Some middles were drawn out

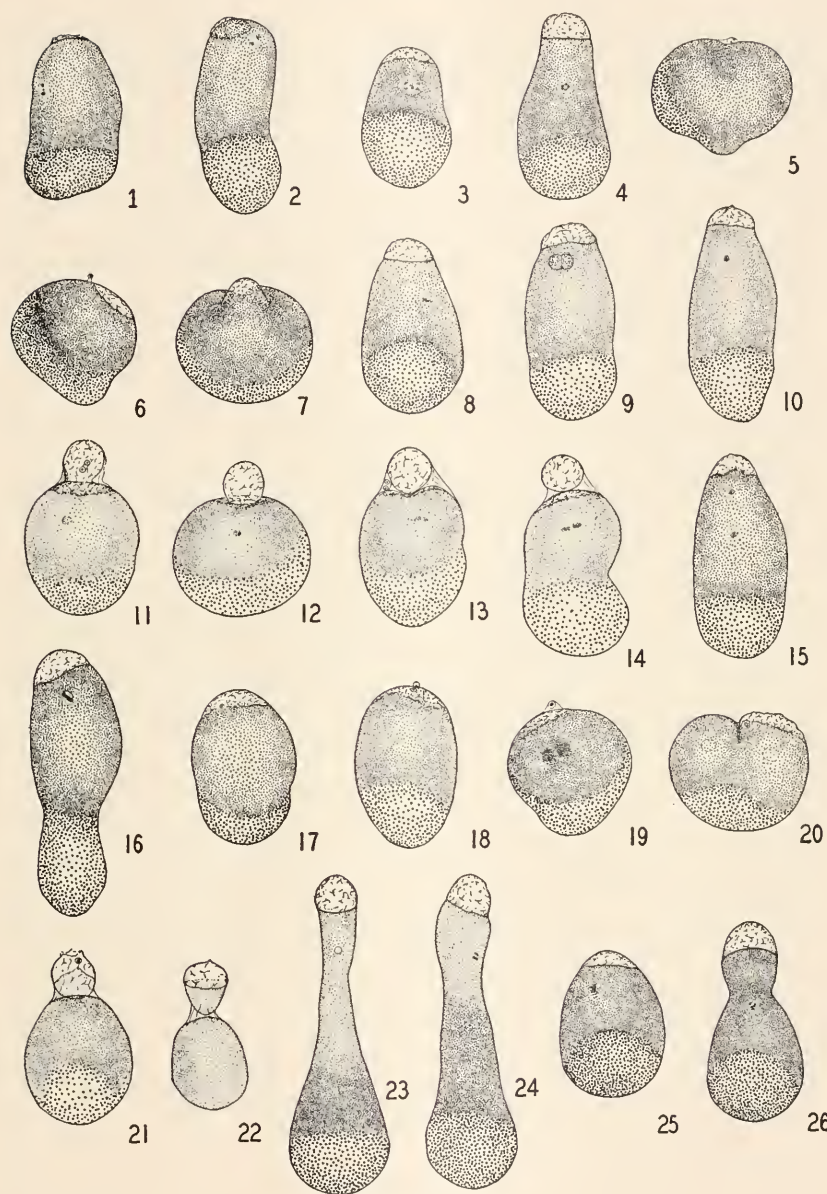


PLATE I

into long strands. In the opposite tube the eggs were taken off after 20 minutes. They were elongated and fewer were broken apart (Figs. 29, 30, 31). They were then fertilized at 9.51, i.e. after two minutes. Seven minutes later they were round. Eleven minutes later a polar body was not present in most eggs. The eggs began to divide after 23 minutes (Figs. 32, 33, 34, 35) but did not show any polar

bodies as a rule. *Comment:* This set of eggs was not fertilized before centrifuging. The eggs were put in the centrifuge 6 minutes after removal. At the time the germinal vesicle was just breaking down. They were centrifuged for 20 minutes. Later, when fertilized, they did not give off a polar body, or perhaps rarely. The meiotic spindle did not bring about division of the eggs.

(10) Unfertilized eggs stood in sea water four minutes; they were then centrifuged for 15 minutes at 2080 r.p.m. The eggs were much elongated. Fertilized, they contracted, some into two parts (as shown in Fig. 36). Later, some of them had a polar body at the side, and later still divided with an antipolar lobe. Eggs in the opposite tube were centrifuged at the same rate for 10 minutes longer. They were more elongated, and many consisted of two bulbs connected by a narrow neck; others were broken apart. The eggs were fertilized, and in the course of 10 minutes most of them had contracted into an oval form; but a number of them contracted into two spheres joined by a narrow neck. Preparations of these showed that the two parts were not the result of a division, as shown by the position of the chromosomes in the outer sphere and the presence of a polar body at the surface (Figs. 36, 37, 38). *Comment:* This experiment shows that even when the eggs were elongated at the time of fertilization the meiotic spindle did not divide the egg. If by chance it had happened to lie in the neck, it is possible that conditions there might have led to the separation of the anaphase plates, but if this should happen the division of the protoplasm into two parts would not be due to the meiotic figure but rather to the contraction of the distal and proximal parts, or bulbs of protoplasm and the breaking of the connecting strand.

(11) Unfertilized eggs were put into the centrifuge tubes one minute after removal when the germinal vesicle was still intact, and rotated for 5 minutes at 1110 r.p.m. The contents had begun to be stratified and the germinal vesicles had disappeared. After killing in picro-acetic, the sections were stained in iron haematoxylin and safranin. A broad oil cap is present, followed by a clear zone (stippled in Fig. 39). The centrifugal end of the egg has a clear faintly coagulated reddish sphere. A deeply-stained, blue crescent of finely granular material lies over it. Centrifugal to this clear area is a half circle of large, deeply-stained granules. The outermost layer is stained red and is without obvious granules. The scattered chromosomes lie in the clear zone, about the middle of the egg, and just above the blue crescent. A large black nucleolus lies somewhere in the same region. Beneath the egg-membrane there is a layer of minute red granules. I do not know what material makes up the blue crescent, perhaps chondriosomes. The deeply-stained black granules may be called yolk, which leaves unnamed the red outer layer of the distal end in which the black granules are imbedded. Some of the remaining eggs were fertilized 5 minutes later. The extrusion of polar body was little delayed, if at all, in these eggs. It appeared most often at the edge of the oil field or even in its center. Later most of the eggs divided, but most of them showed only one polar body in the preparations.

Half of the same lot of eggs were centrifuged for 5 minutes more at 1110 r.p.m. (Fig. 40). They gave nearly the same picture as before (Fig. 39), except that the chromosomes are collecting around an aster, and the nucleolus has disappeared. The oil area is smaller and, after fertilization, the polar body area lies outside the oil field. The extrusion of the polar body was delayed (8 minutes). Later some eggs were found to have two polar bodies.

(12) Unfertilized eggs of another lot were put at once in the centrifuge and rotated for one-half hour at 2080 r.p.m. Many eggs were broken. The preserved, intact eggs, sectioned and stained in Delafield's haematoxylin, showed four distinct layers with the chromosomes arranged around a clear body, probably the nucleolus, in the center of the inner stained zone, or at its surface. No mitotic spindle was found in either case. The rest of the eggs were centrifuged one-half hour longer. The conditions were practically the same as in the preceding set. In both cases some eggs were later fertilized. One or two polar bodies came off, and later most tops and whole eggs divided.

It will be noticed that when the eggs are centrifuged at once the germinal vesicle breaks down, setting free the chromosomes in the middle layer of the egg. Remaining on the centrifuge for one-half to one hour the group of chromosomes either stays in place, or is carried or moves to the surface. The spindle must develop later in some eggs, at least, if they are fertilized, since a polar body may be given off. In other cases the chromosomes remain in the interior, possibly because the egg itself has passed to a later stage of its development, or because the meiotic spindle does not develop, or because if it does, it fails to carry the chromosomes to the surface. Also to be taken into account is the fact that the mass of eggs is crowded together at the end of the centrifuge tube where a lack of oxygen may slow down the development. In such cases the whole egg would be expected to be held back, and then resume its processes at the stage where it was stopped.

(13) The eggs were allowed to stand for 15 minutes in sea water. The polar spindle would then be at or near the surface. The eggs were centrifuged for 15 minutes at 2080 r.p.m. Most of them were killed in Flemming, sectioned, and stained in haematoxylin and eosin. As shown in Fig. 41 five zones are easily recognized in the somewhat elongated egg. The oil cap is deep; beneath it lies a stained granular zone. The spindle lies in this zone near the surface. Next comes a less stained zone of finely granular material. This is followed by a zone of larger granules that run in streaks as though moving outward. Then follows a zone of large granules or lumps buried in a less stained material (probably the red stained material of eggs killed in picro-acetic stained with eosin).

The other half of the same lot were centrifuged 15 minutes more at 2080 r.p.m. The eggs were more elongated (Fig. 42) and showed the same five layers as those of Fig. 41. The distal dark layer is more nearly fused with the outer black layer, but the preparation is so black that the distinction between these two layers is more difficult to see. Later, the centrifuged eggs of this set were fertilized after 40 minutes. Some eggs showed polar bodies at one side (after 15 minutes), then became pear-shaped; a lobe appeared and the eggs divided normally at the normal time. Neither the elongation nor the stratification interfered with normal cleavage.

A Double Egg

One giant egg was found in a series of sections of centrifuged eggs (Fig. 43). It is here drawn to the same scale as the others, but owing to imbedding etc., it is more shrunken. Its double size is shown by comparison with other normal-sized eggs (Fig. 44) on the same slide. The normal eggs show polar spindles in anaphase with 9 chromosomes in each half-plate (Fig. 45). The chromosomes of the giant are scattered in the center of what appears to be a large bipolar spindle (Fig. 46). There are about 18 chromosomes, which is double the normal number, but if they have already divided this would not be the case. There is no real evidence, however, that they have divided as yet, and moved apart.

Fused Eggs

Fused eggs were found in two preparations (Figs. 47-51). The eggs had stood for 3 minutes in sea water, were then fertilized, and after 13 minutes were centrifuged at 1110 r.p.m. for 2 minutes, at 2080 r.p.m. for 5 minutes and at 2750 r.p.m. for 15 minutes. The eggs were

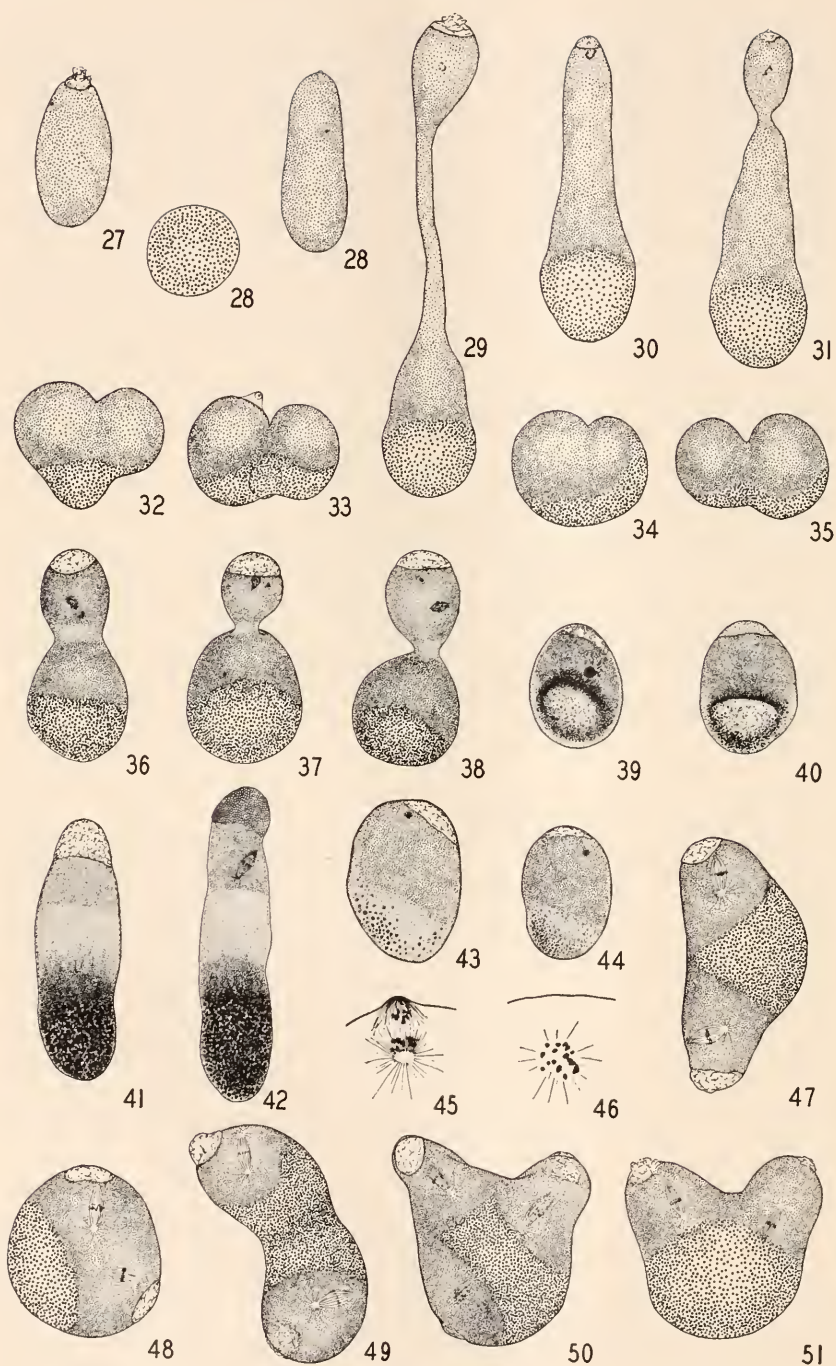


PLATE II

about twice as long as wide. Amongst them were three "fused eggs" (Fig. 50). The yolks are completely fused but the protoplasmic parts are still separated and each contains a polar spindle. These eggs were killed 28 minutes after fertilization. There is one other case in the same preparation in which five eggs are fused, at least they have a large common yolk, the protoplasm and oil of each making different angles with the yolk. The fused eggs must have been stratified before fusing, since the stained zone and oil cap make different angles with the yolk.

There is one other preparation (Fig. 51) that has one double egg. It had been treated in much the same way as the last, but its history is not entirely clear.

Centrifuging the Eggs of Cumingia

When the eggs of *Cumingia* are ejected from the siphon there is present a well-developed spindle that lies somewhat excentrically in the interior of the egg in a more or less horizontal position or else pointed toward the pole. It remains there until the egg is fertilized and then moves to the pole. When the unfertilized egg is centrifuged the spindle remains in the clear zone (see below). After removal to sea water and fertilization, the polar bodies are given off without respect to the stratification, which means that the eggs are little, if at all, oriented in the tubes. The eggs are faintly pink; some lots have more pigment than others. Most of the pigment lies in a broad zone in the outer region of the egg. When centrifuged the oil is quickly driven to the centripetal end. When centrifuged for a longer time (one hour) at a higher speed, the pigment is driven to the end opposite the oil leaving a broad clear middle zone that fills most of the egg. If this zone is carefully examined it is found to consist of a clear zone beneath the oil and a faintly yellowish zone next to the pigment. There is a rather sharp boundary line between these two if the eggs have been sufficiently centrifuged. If the living egg is stained, by adding a little methyl green to the sea water, the zone next to the pigment becomes slightly bluish-green, increasing the difference between it and the protoplasmic zone.

In 1908, 1909, and 1910 I described the results of centrifuging this egg. Four zones were recognized, but the difference between the protoplasmic and yolk zone was not clearly distinguished and what is identified as yolk in the sections (1910, Plate II, Fig. *N-U*) is the pigment plus probably some yolk also. In 1934 Costello recognized four zones identifying the yolk and the pigment as two separate zones. Stronger centrifugation than I had employed was used by Costello, *viz.* 6000 gravity.

Sections of eggs killed immediately show a small clear vesicle just

beneath the surface not previously recognized. Its origin and nature was not determined, but in a set of eggs that had stood three-quarters of an hour in sea water there was observed a clear cap on the eggs surrounded by a faint ring of pigment. Whether this cap is derived from the clear vesicle is not known. This cap had been seen in 1910 and recognized, but such eggs may have stood for some time in sea water.

Sections were made of normal eggs and of those after centrifuging. They were killed in Flemming or in Bouin solutions and stained in haematoxylin and different dyes. The normal egg, after Flemming, contains a number of deeply-stained granules that are scattered through the protoplasm around the spindle, except at one point, the pole of the egg towards which the spindle is pointed. When the living egg is centrifuged these granules are driven to the centrifugal pole where they fill a segment of the egg that is red or pink in color. This region stains yellow after certain killing and staining reagents, and the granules in it are difficult to see and perhaps tend to fuse together. In the earlier paper (1910, Plate II) this region is spoken of as the yolk. Sometimes black stained pigment granules lie more or less scattered in this yellowish "yolk" depending on methods of preparation. Whether there is present in addition to the pigment granules another material in this part of the centrifuged egg in which the dark granules are imbedded is uncertain. The region next to this is distinguished from the clear protoplasmic region only with difficulty in the living centrifuged egg. In sections of stained eggs it can also be distinguished from the protoplasm zone. Whether it is "yolk" material in the ordinary sense, or another kind of substance, or yolk of a somewhat different nature, remains uncertain.

When the living centrifuged eggs are put into Flemming solution after centrifuging the pigment segment immediately becomes brown. The oil segment at the opposite end more slowly becomes brown, but later jet black. It holds this color throughout the treatment with the reagents subsequently used (water, alcohol, xylol, etc.), but the pigment section loses its dark stain and may stain a light yellow if picric acid is present or stains red in eosin. In some of my earlier papers (1910) the pigment region of the centrifugal hemisphere of the living egg is colored red. These eggs were not centrifuged as long or at as great a speed as were those mentioned above, where the pigment is driven further into the centrifugal pole to form there a red segment. On the other hand, in some of the stained sections (Plate II) there is a yellow segment that is spoken of as yolk and no dark granules are indicated. As stated above, this "yolk" is either the pigment granules that have

coalesced in the preserving reagent, or a substance in which they are imbedded.

Drawings of other sections of eggs (in that paper) that were probably preserved in another reagent (Flemming), show the pigment granules scattered in the protoplasm before centrifuging; and after centrifuging they are represented, as here, driven to the centrifugal region.

The presence of a large mitotic spindle in the interior of the *Cumingia* egg suggested that the egg might be a suitable object to examine whether the spindle could be induced to divide the egg, producing a "giant polar body." However, since this spindle and its equatorially arranged chromosomes do not become active until a spermatozoön enters the egg, it becomes necessary to fertilize the egg either before or after centrifuging to activate the spindle. Both procedures have been used, but with two possible exceptions no such division was brought about.

Eggs centrifuged before fertilization, removed to sea water and then fertilized give off at least one, and in most cases probably both polar bodies. Eggs centrifuged after being fertilized give off polar bodies either on the machine or after removal. The length of time in the centrifuge tube was also taken into account, since eggs that were not lying in the axis of centrifuging or even inverted would be expected to behave differently if the centrifugal force was sufficient to keep the polar spindle from moving to the true pole. If, on the other hand, the pole should lie at the more centripetal end of the protoplasmic zone, the movement of the spindle and its surrounding rays might even be facilitated while on the centrifuge. In the earlier experiments that I made (1910) on the egg of *Cumingia* I found that the polar spindle may push its way through the yolk or pigment or oil and give off the polar bodies at the surface. This means that neither of these regions presents a serious obstacle to the movements of the spindle, although statistical evidence is lacking as to whether to some extent there may be some difficulties in reaching the pole when the yolk underlies it. Moreover, in these cases the centrifuging was low, and not long enough to separate visibly the pigment and yolk fields. If centrifuged at a higher rate, or for a longer time, the outcome might have been different.

A few examples may be cited as follows: Unfertilized eggs were centrifuged for 15 minutes at 1664 r.p.m. at $10\frac{1}{2}$ cm. from the center of rotation. When removed the eggs were not elongated or very little so. Fertilized after 5 minutes many eggs were seen to be giving off a polar body after 15 minutes at the side of the clear zone. No giant polar bodies were formed. A few eggs about twice the size of others were recorded in this set.

Another set was fertilized and stood for 12 minutes in sea water and

was then centrifuged for 10 minutes at 1664 r.p.m. They were spherical or columnar, and a few showed a dent at the side. After 6 minutes some eggs extruded a polar body. Only a few eggs divided; also the controls were not normal.

One set centrifuged as above was then fertilized. Eight minutes later polar bodies were observed, some of them exceptionally large. No second polar body was observed 7 minutes later, but 5 minutes later one polar body was seen in some eggs and rarely two. A deep constriction was observed in some eggs at this time. The eggs divided 10 minutes later.

The following cases apply to eggs centrifuged after fertilization. Since the polar spindle is active only after fertilization it is here rather than in the preceding cases that we might hope to bring about a division of the egg by means of the polar spindle, especially when the eggs were kept on the centrifuge during the active phase of the spindle.

Four minutes after fertilization the eggs were centrifuged for 20 minutes at 1664 r.p.m. The first polar body is expected about 20 minutes after fertilization. Two minutes after removal an egg was found divided into two nearly equal parts. This is one of the two cases observed that might be interpreted as due to the polar spindle dividing the egg. Nine minutes later polar bodies were observed in some eggs. Eleven minutes later the eggs divided. In the control, normal eggs, the first polar body came off 21 minutes after fertilization. There was, then, some delay in polar body extrusion in the centrifuged eggs.

Another set stood for 10 minutes after being fertilized. They were then centrifuged for 12 minutes at 3260 r.p.m. When removed the first polar body had been given off while in the centrifuge tube in some eggs. Later, when the eggs were divided, one, two or no polar bodies were found.

Another set was fertilized and allowed to stand until the first polar body was out ($18\frac{1}{2}$ minutes). This was an attempt to find out whether the second polar spindle was more efficacious in dividing the egg than the first one. The eggs were centrifuged for 28 minutes at 1664 r.p.m. They were then a little oval, and some showed a slight constriction. No divisions were present.

These few experiments are only exploratory. The negative results, with two possible exceptions, can not be understood to exclude the possibility that under other conditions the polar spindles would divide the egg, but the experiments were not promising.

Morgan and Tyler, in 1935, centrifuged the eggs of *Urechis* under conditions similar to those described here. In one respect the egg is different from those of *Chaetopterus* and *Cumingia*; the germinal vesicle

remains intact until the eggs are fertilized; it then breaks down, and the dent in the egg disappears. Eggs centrifuged before fertilization give off the first polar body at the true pole and produce normal embryos. If centrifuged after fertilization the chromosomes are driven towards the pigment side of the egg (centrifugally), and the polar bodies are given off somewhere in that hemisphere; if they are given off at all. The evidence indicates that these eggs fall at random on the machine, hence in many cases the polar bodies are given off at other regions of the egg than that of the true pole, as Conklin also found in *Crepidula*. The evidence also shows that the first and second cleavage planes pass through the secondary pole, but these eggs do not produce normal embryos. It can not be said, therefore, that a new polarity has been imposed on the egg, in so far as "polarity" is an essential feature of development.

When the eggs are on the centrifuge at the time when the chromosomes are set free from the germinal vesicle and the polar spindle is in process of formation, separate asters may appear in the same regions as the chromosomes. Later a spindle may develop between two asters and these are the eggs that give off polar bodies. When, on the other hand, the chromosomes remain in the egg, most of them are collected on the cleavage spindle; but some may remain outside attached to the astral rays. The abnormal development of the embryo may, in part, be due to the irregular distribution of the chromosomes.

If eggs are centrifuged after the extrusion of the first polar body, the second polar spindle may be displaced from the pole, but does not bring about the division of the egg. Whether it returns to the pole or gives off a polar body at some other point was not definitely settled, although in a few cases two polar bodies were observed at different points on the surface. In none of these experiments were the eggs kept long enough on the centrifuge to pass by the time when the polar bodies are formed in the normal egg. In this respect the experiments with *Chaetopterus* and *Cumingia* cover somewhat different grounds.

BIBLIOGRAPHY

- CLEMENT, ANTHONY C., 1935. The formation of giant polar bodies in centrifuged eggs of *Ilyanassa*. *Biol. Bull.*, 69: 403.
- CONKLIN, EDWIN G., 1915. Why polar bodies do not develop. *Proc. Nat. Acad. Sci.*, 1: 491.
- CONKLIN, EDWIN G., 1916. Effects of centrifugal force on the polarity of the eggs of *Crepidula*. *Proc. Nat. Acad. Sci.*, 2: 87.
- CONKLIN, EDWIN G., 1917. Effects of centrifugal force on the structure and development of the eggs of *Crepidula*. *Jour. Exper. Zool.*, 22: 311.
- LILLIE, FRANK R., 1906. Observations and experiments concerning the elementary phenomena of embryonic development in *Chaetopterus*. *Jour. Exper. Zool.*, 3: 153.

- LILLIE, FRANK R., 1909. Polarity and bilaterality of the annelid egg. Experiments with centrifugal force. *Biol. Bull.*, **16**: 54.
- LILLIE, FRANK R., 1909. Karyokinetic figures of centrifuged eggs; an experimental test of the center of force hypothesis. *Biol. Bull.*, **17**: 101.
- MORGAN, T. H., 1908. The effects of a centrifugal force on the eggs of Cumingia. *Science*, **27**: 446.
- MORGAN, T. H., 1909. The effects produced by centrifuging eggs before and during development. *Anat. Rec.*, **3**: 155.
- MORGAN, T. H., 1910. Cytological studies of centrifuged eggs. *Jour. Exper. Zool.*, **9**: 593.
- MORGAN, T. H., 1927. Experimental Embryology. Columbia University Press. New York.
- MORGAN, T. H., 1933. The formation of the antipolar lobe in Ilyanassa. *Jour. Exper. Zool.*, **64**: 433.
- MORGAN, T. H., 1935a. Centrifuging the eggs of Ilyanassa in reverse. *Biol. Bull.*, **68**: 268.
- MORGAN, T. H., 1935b. The separation of the egg of Ilyanassa into two parts by centrifuging. *Biol. Bull.*, **68**: 280.
- MORGAN, T. H., 1935c. The rhythmic changes in form of the isolated antipolar lobe of Ilyanassa. *Biol. Bull.*, **68**: 296.
- MORGAN, THOMAS HUNT, 1936. Further experiments on the formation of the antipolar lobe of Ilyanassa. *Jour. Exper. Zool.*, **74**: 381.
- MORGAN, T. H., 1937a. The behavior of the maturation spindles in polar fragments of eggs of Ilyanassa obtained by centrifuging. *Biol. Bull.*, **72**: 88.
- MORGAN, T. H., 1937b. The factors locating the first cleavage plane in the egg of Chaetopterus. *Cytologia, Fujii Jubilee Vol.* p. 711.
- MORGAN, T. H., 1938. A reconsideration of the evidence concerning a dorso-ventral pre-organization of the egg of Chaetopterus. *Biol. Bull.*, **74**: 395.
- MORGAN, T. H., AND ALBERT TYLER, 1935. Effects of centrifuging eggs of Urechis before and after fertilization. *Jour. Exper. Zool.*, **70**: 301.
- MORGAN, T. H., AND ALBERT TYLER, 1930. The point of entrance of the spermatozoön in relation to the orientation of the embryo in eggs with spiral cleavage. *Biol. Bull.*, **58**: 59.
- TYLER, A., 1930. Experimental production of double embryos in annelids and mollusks. *Jour. Exper. Zool.*, **57**: 347.
- WHITAKER, DOUGLAS, AND T. H. MORGAN, 1930. The cleavage of polar and antipolar halves of the egg of Chaetopterus. *Biol. Bull.*, **58**: 145.
- WILSON, EDMUND B., 1929. The development of egg-fragments in annelids. *Arch. f. Entw.-mech.*, **117**: 179.
- WILSON, EDMUND B., 1930. Notes on the development of fragments of the fertilized Chaetopterus egg. *Biol. Bull.*, **59**: 71.

THE REPRODUCTIVE CYCLE OF THE VIVIPAROUS TELEOST, NEOTOCA BILINEATA, A MEMBER OF THE FAMILY GOODEIDAE¹

I. THE BREEDING CYCLE

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INTRODUCTION

Much attention has been devoted recently to the reproductive cycles of viviparous teleosts. Some of the recent work has been concerned with the histological phases of the gonad during reproduction (Scott, 1928; Bailey, 1933; and Turner, 1933, 1938*b*) but more attention has been devoted to different aspects of the breeding cycle. In this respect, much of the work has been concerned with members of the family Poeciliidae (order Cyprinodontes) because of the large number of viviparous species available and because of their relative abundance (see Turner, 1937*a* for recent work on the poeciliids). With the exception of three papers (Turner, 1933, 1937*b*; Mendoza, 1937) nothing more has been reported on the family Goodeidae from the central plateau of Mexico, a family which contains twenty-six viviparous species. In view of the dearth of information on the goodeids, this investigation was undertaken to study the female reproductive cycle of *Neotoca bilineata* in order to throw some light on the reproductive activities of this viviparous family. The information obtained in this investigation involves three phases of the reproductive activity of *Neotoca*: (1) the breeding cycle, (2) the germ cell cycle during gestation, and (3) the somatic cycle of the ovary. The present paper is devoted entirely to the phenomena associated with breeding as observed in the laboratory: the breeding season, the lengths of the brood intervals, the sizes of broods, superfetation, fertilization, and color variations in the female. The periodic variation of the germ cells in the ovary and the gross and microscopic changes which the ovarian soma undergoes during gestation will be dealt with in later papers.

¹ I wish to express my appreciation for the advice and guidance of Dr. C. L. Turner under whose direction this investigation was undertaken.

MATERIALS

This form was chosen because of the number of females available, their convenient size, and because of the rapidity of brood production. Specimens of *Neotoca* used during the investigation were raised in the laboratory. A record of over eighty females was kept during the course of the investigation; approximately thirty-five of these were killed and serial sections made of the ovary for microscopic examination. No single female was kept during the entire period of observation, which lasted from the fall of 1934 to the spring of 1937, since specimens frequently died or were killed during critical periods of the year and were replaced by new females. The individual females under observation always were isolated with one or two males, the isolation tanks consisting chiefly of one- or three-gallon aquaria. All tanks were filled with seasoned water and abundantly stocked with plants. With the exception of a few aquaria, all were kept in the same room, subject to a fairly constant room temperature of about 70° F. and the normal, daily fluctuation of light. Electric lights were turned on but infrequently at night so that any possible effects of artificial illumination on the reproductive cycle were negligible. Records were kept of the date of isolation of the females, frequency of brood production, number and size of young, and the condition of the maternal gonad at time of death. In most cases, when females were killed a unilateral dissection of the ovary was made and measurements of the young on that side were taken; the opposite side of the ovary was left intact for sectioning. In a few females, the entire ovary was sectioned without the unilateral dissection.

BREEDING SEASON

The following description of the breeding season is based solely on laboratory observations; there has been no study of the females in their natural habitat such as was performed by Scott on *Fitzroyia lineata* (1928) and by Turner on *Brachyrhaphis episcopi* (1938a).

Table I shows the preponderance of broods appearing during spring and early summer. Series A consists of older females isolated during the early fall of 1934. Of the nineteen females isolated, only six had October broods and only one of the six started further gestation that fall. The other five specimens already had started a period of sexual inactivity or rest destined to last until the following spring. The remaining females of this series were allowed to live through the fall and winter but they produced no more broods; nevertheless, the following spring all but one of the females started to breed again. Evidence of a period of

reproductive inactivity over the winter is obtained from the fact that over half of the females in Series A had no fall broods. Additional evidence of this winter inactivity appeared constantly during the investigation.

Early in 1935, the individual females of Series B were isolated and records of brood production were started. It is evident from Table I that but few broods were produced during January and February; however, almost all females produced broods during March and April. During May there was a decrease in the number of broods followed by a further decrease during June and July. The latter month marks the termination of brood production. Females that were alive after July bore no young during the summer, fall or winter.

The females of Series C were isolated during the fall of 1935. With the exception of one specimen which had young during December, no other broods were produced until March. It is evident again that broods appeared predominantly during spring and early summer. July again marks the termination of brood production.

Series D comprises a small group of females isolated during the fall of 1936. Of about one dozen females isolated, only three had had young by February of 1937 at which time the records were discontinued.

For some reason not understood, many deaths were recorded during the months of May, June, and July whereas deaths during other times of the year were most infrequent. The marked mortality evident in the females so jeopardized the regularly breeding specimens that frequently they were sacrificed during July and preserved rather than to risk death and subsequent loss of the tissues for histological examination. If it had not been desired to get a complete histological record of the ovary during gestation, females would have been permitted to live as long as possible so as to get a continuous record of breeding throughout various seasons. It is possible then that despite the deaths of some females, few would have produced a small number of broods during summer, fall, and winter. The total number of broods produced during each month over a period of three years was as follows:

Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.
6	10	19	29	18	14	10	0	0	4	2	1

In summary it may be said that brood production occurs chiefly during spring and early summer whereas the greatest number of broods appears during the month of April. The months of August and September show no brood production. This may be caused in part by the fact that most females had been killed or had died by the latter part of July. Among the females that remained, however, no brood production

was observed. It is very likely that, had older females been used, more fall broods would have been recorded.

BROOD INTERVALS

Some viviparous fishes such as *Poecilistes pleurospilus* and *Heterandria formosa* (Turner, 1937a) are characterized by a marked decrease

TABLE I

The complete record of breeding from the fall of 1934 to the early spring of 1937. The number of the different females is merely for purposes of identification. The columns marked "Brood I," "Brood II," etc., indicate the dates of the first, second broods etc.; the dates are expressed as fractions, the numerator indicates the month of the year, the denominator indicates the day. The columns marked "Interval" include the interval of days between successive broods. Days are recorded on which the females were killed or died naturally; the asterisk following the date of death indicates that the female was bearing young at the time.

Female		Brood I	In- terval	Brood II	In- terval	Brood III	In- terval	Brood IV	Female killed	Female died	
	1	10/1							11/12*		1934
	2	10/8							11/12		
	3	10/11							11/15		
	4	10/22							11/19		
	5	11/7							11/21		
	6	11/14							11/21		
Series A	8								2/6*		1935
	9								2/6*		
	10								2/6*		
	11								2/6*		
	12								2/6*		
	13								2/6*		
	14								2/6*		
	15								2/23		
	16	2/15							3/4*		
	19	3/9							4/1		
Series B	1	1/8	62	3/11						5/7	1935
	2	1/29	50	3/20	15	4/4				5/10	
	3	2/2	44	3/17	40	4/26	39	6/4		7/4	
	4	1/1									
	5	5/29									
	6	2/10	71	4/21	48	6/8				7/9	
	7	2/13	55	4/8					4/26		
	9	2/15	45	3/31	41	5/11					
	10	2/15	17	3/3						5/17	
	11	3/4	43	4/16	41	5/27					
	17	3/11	45							5/4	
	18	3/10	66	5/16						6/3	
	19	3/21	40	4/30	40	6/9	40	7/17	7/29*		
	20	3/16	41	4/26	44	6/9	40	7/19	7/24		
	21	3/23	36	4/28	44	6/11				7/17	
22	3/23	42	5/4	36	6/10	40	7/20	8/5*			
23	3/27	43	5/9	48	6/26			7/21*			

TABLE I (*continued*)

Female		Brood I	In- terval	Brood II	In- terval	Brood III	In- terval	Brood IV	Female killed	Female died	
Series B	24	3/27	51	5/17	54	7/10			7/21	1935	
	25	4/1	41	5/11	44	6/24					
	26	4/19							7/20		
	27	4/5	42	5/17	44	7/1			7/21*		
	28	4/6									
	29	4/6	31	5/7							
	30	4/9	48	5/27							
	31	4/9									
	32	4/9	45	5/24							
	33	4/16									
	34	4/15	46	6/1							
	35	4/20									
	36	4/25	40	6/4	45	7/19					
	37	4/24									
	38	4/26									
	40	5/28									
	43	5/21									
	44	5/13									
	46	6/3	47	7/20							
	47	7/19							8/11		
Series C	48	3/23	60	5/23						1936	
	49	12/1	120	4/29	64	7/2					
	56	4/23	49	6/11					6/19*		
	57	3/1	55	4/25							
	59	6/1	39	7/9					7/16		
	60	3/23	43	5/6							
	63	5/6									
	66	6/20							6/20		
	67	3/7									
	72	4/8	49	5/27							
	73	3/1	41	4/17					6/14*		
Series D	76	1/15								1937	
	77	1/23									
	81	1/12									

in length of brood intervals with the advance of the breeding season. Intervals between broods decrease from as much as twenty-six to twelve days in the former and from forty-one to three or four days in the latter. In *Neotoca*, however, as the breeding season advances there is no apparent increase in the rapidity of brood production. Furthermore, the average length of the brood intervals occurring during different months of the breeding season show no significant difference in length throughout the season (Table II).

Most brood intervals are regular and fluctuate around a normal fixed period. Sixty-eight per cent of the 51 recorded intervals show a maximum variation of 9 days (39–48 days) whereas fully 51 per cent of the intervals vary but 5 days (40–45 days). The numerical average for

TABLE II

Average length of brood intervals throughout the breeding season.

Time Occurrence of Intervals	Average Length of Intervals
	<i>days</i>
February–March	43.6
March–April	43.8
April–May	47.3
May–June	43.4
June–July	45.8

the entire group is 45.0 days. Although most intervals are fairly regular, a few variations ranging from 15 to 71 days were recorded. As an example of regularity in brood production, the following case is cited (Table III). It involves the parallel breeding behavior of four females (Series B) occupying adjacent tanks.

TABLE III

The breeding record of four females in adjacent tanks.

Female	Brood I	Int.*	Brood II	Int.	Brood III	Int.	Brood IV
19	March 21	40	April 30	40	June 9	40	July 19
20	March 16	41	April 26	44	June 9	40	July 19
21	March 23	36	April 28	44	June 11		
22	March 23	42	May 4	36	June 10	40	July 20

* Interval in days.

In addition to the above example of parallel breeding, several other instances led the writer to suspect that breeding was unusually plentiful on certain days, as if there were regular waves of brood production among several females. Analysis of the brood record resulted in the following interesting conclusions. There occurred three distinct groups of females, with members of each group having parallel breeding cycles. The breeding period for each group normally lasted about ten days and appeared again approximately forty-five days later. The number of females in each group varied from four to eight individuals, each female producing two or more broods. Each group retained its identity despite the fact that members within a group did not produce their broods in any regular order. The groups furthermore retained the same relative

order in their breeding periods throughout the season: *ABC*, *ABC*, *ABC*, *AB*, *A*. The groups were not composed entirely of females starting brood production during the same month; some females started breeding later in the season and yet their cycle corresponded with one of the group cycles already established. Of a total of 80 broods produced during 1935 between January 1 and July 21, 58 fit into this regular cyclic phenomenon; 13 broods could not be accounted for in this scheme since they were first and only broods; finally, only five females producing a total of 9 broods participated in irregular cycles which failed to fit into the above rhythmic activity. The breeding records other than those of 1935 were too scanty to permit such an analysis.

It is true that the selection of groups was made arbitrarily. There is probably nothing that determines rigidly the number of days during which members of a group will produce young, but segregation of the females was made possible because, regardless of the order of brood

TABLE IV

Increase in size of broods during the breeding season.

Brood	Total number of broods	Total number of young	Average number of young per brood
First.....	53	302	6.0
Second.....	30	240	8.0
Third.....	15	163	10.8
Fourth.....	4	52	10.4

production within the units, the breeding period of one group did not overlap with that of another group. Regularity was maintained remarkably well during the season for a phenomenon which frequently is considered to be sporadic. Finally, the determination of these groups is not significant in itself; rather, the importance lies in that they demonstrate markedly the regularity of the breeding behavior of the females of this species. It is true, obviously, that there are minor differences in the rate of breeding among the individual females.

SIZE OF BROODS

The present analysis of the size of broods is based on records obtained from 53 females which produced a total of 103 broods. On the basis of the information available, it is evident that the broods increase in size during the season. This increase is apparent in the general averages of all first, second, third, and fourth broods (Table IV).

It is emphasized that the above evidence concerns in the main part young females producing their first broods; consequently, the increase in the average number of young per brood for successive broods actually implies that with growth of the females, broods become larger. Although the third and fourth broods were not as numerous as would have been desired, together they indicate the tendency for more numerous young in the third and fourth broods. The difference between the last two broods obviously is too small to be significant. It is to be expected that older females would have produced somewhat larger broods. Only a maximum of four broods is recorded for any one female. It is likely that females have more than four broods during their active reproductive life, but it is doubtful if they have more than four or five broods during one season since the interval between broods is so long.

SUPERFETATION

An outstanding characteristic of the reproductive cycle of *Neotoca* is the absence of superfetation. This absence is concluded from three sources: the brood interval, the evacuation of young, and examination of the ovary itself. Broods are spaced far apart and are regular in their appearance. Furthermore, there is no evidence that normally only a part of the young are evacuated at one time. Generally, young are known to be born within minutes of each other. In only two females was there evidence of a phenomenon resembling superfetation. In both cases the first brood was small and was followed in two weeks by another small brood. Since it is improbable that the young were completely developed in that short a time, it is more likely that they were part of the first brood but for some unknown reason were not expelled along with the others. Since this phenomenon failed to appear elsewhere no significance was attached to its occurrence. Examination of many ovaries, both by gross and microscopic methods, failed to reveal the presence of more than one brood in the ovary at one time. Normally, all embryos in an ovary were in the same stage of development. It is true that slight irregularities in size occurred but they were seldom great. Therefore, it may be concluded that each brood is distinct in *Neotoca* because of the brood interval, the simultaneous evacuation of all young, and because examination of the ovaries in a large number of females revealed but one brood in the ovary at one time.

FERTILIZATION

In viviparous fishes producing annual broods, copulation and fertilization may occur at the beginning of the breeding season or, as occurs

in *Cymatogaster aggregatus* (Eigenmann, 1892), copulation may take place several months before the breeding season, in which case the sperm are stored in the ovary until time for fertilization. Among viviparous forms producing several broods during one season, the time of copulation may vary. In forms such as the poeciliids, there is a continual introduction of spermatozoa by the male. However, storage of sperm has been reported for *Xiphophorus helleri* (van Oordt, 1928; Bailey, 1933) and has been determined experimentally in *Heterandria formosa* by Turner (unpublished data). In such cases, spermatozoa are stored and are capable of fertilizing several sets of ovocytes over a considerable period of time. Normally, however, it appears that the storage is not necessary because of the continual introduction of fresh spermatozoa by the male. There is no evidence that such a storage phenomenon occurs in *Neotoca*; in this species, copulation and fertilization occur concurrently or nearly so. Spermatophores which have been reported for some viviparous teleosts likewise do not occur in this species. The actual time during which fertilization occurs has been determined as follows. Examination of 7 ovaries with young in late stages of gestation failed to reveal spermatozoa in the ovary or any indication of fertilization. Three females killed at intervals of 5, 6 and 7 days after the birth of young likewise failed to reveal signs of fertilization. However, 5 females killed at intervals of from 7 to 15 days after the birth of young revealed new embryos in different stages of segmentation. Spermatozoa were evident in almost all of these ovaries. Although it is possible, of course, that fertilization occasionally may occur at a different time, it is concluded from the above evidence that normal fertilization occurs about the seventh day after the release of a previous brood. The details of the cytology of fertilization will be considered in another paper.

FEMALE COLORATION

Peculiar coloration of the females during certain phases of the breeding cycle has been reported by Scott for *Fitzroyia*. Although the regularity and the significance of these color changes have been questioned by some investigators, reference is made herein to a peculiar change in the color of the females. During the first year it was noticed frequently that the abdominal region of the female became a vivid blue whereas normally it is a pale gray. Furthermore, it was noticed that females with parallel breeding cycles assumed this coloration approximately at the same time. The coloration normally lasted a few days and then disappeared. If the female became excited, the color could disappear in a few seconds to return later in a gradual manner. Weekly observations

during the second breeding season indicated that in non-breeding females, the coloration appeared very irregularly whereas among the breeding females some semblance of regularity was observed. In most cases, the color appeared on an average of 10 days after the birth of a previous brood and 33 days before the birth of the following brood. It is significant that two virgin females, isolated from the time of birth, showed the same coloration simultaneously, at frequencies comparable to the cycles of breeding females. Microscopic examination of the virgin gonads, fixed during the color phase of the female, revealed the ovarian soma in a very active, turgid state similar to that of an ovary during early gestation. Thus it may be that this color is correlated in some way to a periodic activation of the ovarian soma, whether or not the female is exposed to males. Furthermore, the time of appearance of the color in the breeding females correlates closely with the time of fertilization, thereby suggesting that there occurs periodically in the ovary an inherent activation usually accompanied by color changes in the female and evidently facilitating copulation and fertilization.

DISCUSSION

Compared with the breeding activities of other viviparous teleosts, *Neotoca* most closely resembles the poeciliids which in general breed actively during the spring and summer and but little during fall and winter. This seasonal wave of reproductive activity occurs in several genera including *Mollienisia*, *Xiphophorus*, *Gambusia* and others. These poeciliids and *Neotoca* obviously stand in contrast to viviparous forms such as *Cymatogaster*, *Fitzroyia* and others which have but an annual brood. As has been suggested by other investigators (Kuntz, 1913; Turner, 1937a), the writer also is of the opinion that the recurrent broods during the season are comparable to several annual broods rather than to one extended annual brood, as suggested by Barney and Anson (1921) for *Gambusia*. The former opinion is shared by the writer since in *Neotoca* there is a definite and complete activation of the ovary including separate copulation and fertilization for every brood.

Whether the laboratory breeding cycle is similar to that of the females in their natural habitat is unknown. The only information available is that obtained at the time the original stock was collected during March by Dr. C. L. Turner. Reproductive activity was present but not at a maximum. It has been shown for *Gambusia affinis*, at least, that the breeding records of the females in the laboratory and in the field correlate closely. On the other hand, Turner (1938a) has found that *Brachyrhaphis episcopi*, a poeciliid, fails to show this alternate high and low level of reproductive activity but breeds continuously throughout

the year in its normal habitat in the Panama Canal Zone. It is more likely that of the two, *Neotoca* resembles *Gambusia* more closely since the temperature and light factors are not quite as constant in its habitat on the Mexican plateau as is true for *Brachyrrhaphis*. It is probable that the information on the breeding cycle of this species will apply to most goodeids although there is one exception known, *Goodea luitpoldi*, a form which is larger, matures more slowly, has greater brood intervals, and larger young.

The cause of the high mortality evident among the females during early summer is unknown. It is unlikely that death normally follows the production of one or two broods as has been reported for another viviparous form, *Comephorus baicalensis* (Dybowski, 1902). It seems more reasonable to assume that the females are at a low physiological level because of their reproductive activities and hence they are more susceptible to minor changes in food, temperature, or perhaps even to minute organisms in the water. Of these, temperature may prove to be the most important.

It has been shown that the breeding cycle remains quite constant throughout the season, differing from forms such as *Pocillistes* and *Heterandria*. It is indeed difficult to explain the cause of such a regular production of broods. It is not likely that external conditions are of major importance in the establishment of the regular cycles in *Neotoca*. However, it may be that food and temperature especially help to bring about the birth of young on some particular day or interval of days if the young are ready or nearly ready for birth. The regular cycles of brood production very likely are governed by some internal, inherent mechanism, a mechanism which is inherited in the species as a whole and probably expresses itself through hormonal control. Once brood production starts, the inherent periodicity expresses itself usually in a regular manner. However, in the absence of careful experimentation, it is unwise to discuss further the relative importance of external or internal factors in the determination of such a marked regularity in breeding activity.

Since the embryos remain in the ovarian lumen until time of birth, the ovary of *Neotoca* may be compared to the uterus of mammals. There is further similarity in that marked periodic activation of the ovarian soma occurs not only in breeding females but also in virgins. A third parallel exists in that fertilization evidently occurs during this period of activation. Consequently, in view of the above comparisons, it is suggested that there occurs in the female of this species a period similar to that of oestrus in the mammalian female.

LITERATURE CITED

- BAILEY, R. J., 1933. The ovarian cycle in the viviparous teleost *Xiphophorus helleri*. *Biol. Bull.*, **64**: 206.
- BARNEY, R. L., AND B. J. ANSON, 1921. The seasonal abundance of the mosquito-destroying top-minnow, *Gambusia affinis*, especially in relation to fecundity. *Anat. Rec.*, **22**: 317.
- DYBOWSKI, B., 1902. Life-history and young stages of the "Fat-Fish" of Lake Baikal. (Abstract) *Jour. Roy. Micr. Soc.*, p. 167.
- EIGENMANN, C. H., 1892. On the viviparous fishes of the Pacific coast of North America. *Bull. U. S. Fish. Com.* (1894), **12**: 381.
- KUNTZ, A., 1913. Notes on the habits, morphology of the reproductive organs and embryology of the viviparous fish *Gambusia affinis*. *Bull. U. S. Bur. Fish.* (1914), **33**: 177.
- MENDOZA, G., 1937. Structural and vascular changes accompanying the resorption of the proctodaeal processes after birth in the embryos of the Goodeidae, a family of viviparous fishes. *Jour. Morph.*, **61**: 95.
- SCOTT, M. I. H., 1928. Sobre el desarrollo intraovarial de *Fitzroyia lineata* (Jen.) Berg. *Anal. Museo Hist. Nat. de Buenos Aires*, **34**, (*Ictiologia*, pub. num. 12).
- TURNER, C. L., 1933. Viviparity superimposed upon ovo-viviparity in the Goodeidae, a family of Cyprinodont teleost fishes of the Mexican plateau. *Jour. Morph.*, **55**: 207.
- TURNER, C. L., 1937a. Reproductive cycles and superfetation in poeciliid fishes. *Biol. Bull.*, **72**: 145.
- TURNER, C. L., 1937b. The trophotaeniae of the Goodeidae, a family of viviparous cyprinodont fishes. *Jour. Morph.*, **61**: 495.
- TURNER, C. L., 1938a. The reproductive cycle of *Brachyrhaphis episcopi*, an ovo-viviparous poeciliid fish, in the natural tropical habitat. *Biol. Bull.*, **75**: 56.
- TURNER, C. L., 1938b. Histological and cytological changes in the ovary of *Cymatogaster aggregatus* during gestation. *Jour. Morph.*, **62**: 351.
- VAN OORDT, G. J., 1928. The duration of the life of the spermatozoa in the fertilized female of *Xiphophorus helleri* Regan. *Tijdschr. d. Ned. Dierk. Vereen.*, **1**: 1.

THE INFLUENCE OF TEMPERATURE ON THE SURVIVAL, GROWTH AND RESPIRATION OF *CALANUS* *FINMARCHICUS*

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Experiments which have been in progress at the Woods Hole Oceanographic Institution on the nutrition of the ecologically important copepod, *Calanus finmarchicus*, in relation to the food cycle of the sea have shed light on the types of food suitable for this species and on the rate of feeding of which it is capable under different conditions (Clarke and Gellis, 1935; Fuller and Clarke, 1936; and Fuller, 1937). Since the temperatures to which *Calanus* is subjected off our coasts vary greatly with the season and with the locality, it was desired to extend the investigation of the survival and growth of this species in the laboratory to include a larger range of temperature than had been possible previously. In the earlier experiments survival of the copepods had generally been poor, and Gross (1937) has expressed the opinion that even short intervals of temperature change, such as are entailed by the removal of the culture dishes from the constant temperature bath for inspection, are seriously detrimental to animals in culture. In the experiments about to be described we therefore proposed not only to test the effect of a variety of temperatures on growth but also to compare the survival of animals kept continuously at constant temperatures with that of other individuals subjected to periodic changes in temperature.

Calanus was shown to exist in the waters off Woods Hole throughout the year 1935-36 and to undergo reproduction during the spring months (Clarke and Zinn, 1937). Yet food organisms were not sufficiently abundant in these waters to meet the nutritive requirements which we have assumed for *Calanus* even at the maximum rate of feeding observed in the laboratory, if diatoms alone are considered (Fuller and Clarke, 1936; and Fuller, 1937). Food in the form of diatoms appeared to exist in only one-tenth the necessary concentration. This discrepancy has led to such suggestions as the considerable use of the nannoplankton by the copepods and this possibility should be investigated further, but the difficulty could be removed equally well if the

¹ Contribution No. 214.

value assumed for the food requirements of *Calanus* were shown to be ten times too high. The value used was that given by Marshall, Nicholls and Orr (1935) and was calculated from the amount of oxygen taken up by a given number of copepods in unit time. The confirmation of this work was therefore of the greatest importance and we proposed in the present undertaking to repeat the measurements of these investigators, using their own method, and to check them by employing a more precise method. Furthermore, we desired to ascertain whether differences in temperature exert any significant effect on the oxygen requirement of *Calanus* and its application to the nutrition of this species.

SURVIVAL AND GROWTH

Material and Methods

For the first group of experiments, which was conducted at Woods Hole during the summer of 1937, copepods were obtained from off-shore localities in the vicinity of Cape Cod. In the second group of experiments, which was undertaken at the Harvard Biological Laboratories in Cambridge, material was obtained off Gloucester. In all cases the copepods were collected by towing a scrim net for a few minutes, and were placed in jars which were kept cold during the trip to the laboratory. Immediately upon arrival the animals were transferred by means of a pipette to covered culture dishes and these were set in baths whose temperature was maintained constant to within 0.5° C. In all the experiments except those involving a change of temperatures (see below) the copepods were transferred each day by pipette to freshly prepared culture dishes (each containing 250 cc. of water) which had been brought to the proper temperature.

The sea water used in the Woods Hole experiments was taken freshly from the harbor (except for the experiments of Table II); that used in Cambridge had been collected at Nahant several months previously and allowed to stand in carboys. In both instances the water was Berkefeld filtered (except for a few tests at Woods Hole in which unfiltered harbor water was used) and then thoroughly aerated.

In most of the experiments a known concentration of food was provided by adding a measured amount of a "persistent" culture of either the diatom, *Nitzschia closterium* (Plymouth strain) or the green flagellate, *Platymonas subcordiformis*² to each culture dish. The amount of organic matter represented by these forms and also by the diatom, *Rhizosolenia* sp., which was abundant in Woods Hole waters during the

² Kindly identified for us by Professor G. W. Prescott of Albion College, Michigan.

summer, may be judged from the following determinations of nitrogen kindly carried out for us by Dr. T. von Brand:

<i>Nitzschia</i>	2.5	γ N	per	10^6	cells ³
<i>Platymonas</i>	12.7	"	"	"	"
<i>Rhizosolenia</i>	96.0	"	"	"	"

To ascertain whether the number of animals present or the concentration of food organisms was sufficient to affect significantly the dissolved oxygen or the pH of the culture medium, tests were carried out on a representative series of culture dishes as indicated in Table I. The

TABLE I

Tests of pH and O₂ in culture dishes with different numbers of copepods and of food organisms.

Concentration of <i>Nitzschia</i> cells/cc.	Number of Stage V <i>Calanus</i>	pH		O ₂ cc./liter	
		Initial	After 24 hrs.	Initial	After 24 hrs.
0	0	8.09	7.86	4.25	5.67
0	10	8.09	7.81	4.25	6.42
30,000	0	8.11	9.09*	4.68	5.94
30,000	10	8.11	7.73	4.68	6.65
300,000	0	8.17	8.10	4.81	6.49
300,000	10	8.17	8.00	4.81	6.37

* This value shown by other tests to be atypical.

results show that sufficient O₂ was present in all containers and that the pH was only slightly altered. The slight increase in O₂ content and decrease in pH in the control was due to incomplete temperature equilibrium initially.

Experiments

The first set of experiments was designed to ascertain whether the change in temperature which results from the removal of the culture dishes from the constant temperature bath for daily inspection and transfer of animals has a deleterious effect on the copepods. For this purpose groups of *Calanus* and also of *Centropages typicus*—another copepod common in the Woods Hole region—were divided into two parts and placed in culture dishes in constant temperature baths maintained at either 8° C. or 13° C. One culture dish in each group was left undisturbed in the bath as a control but others were removed for varying lengths of time as indicated in Table II. For the duration of

³ The reason why this value is larger than that found the year before and reported by Fuller (1937) is not known. Variations of this sort must be taken into consideration in calculating the potential food value of each species.

the experiments the survival of *Calanus* was better than that of *Centropages* but in neither case was survival in the control dishes significantly better than that in the dishes which were subjected to a daily temperature change. We conclude, therefore, that contrary to the suggestion of Gross (1937), the daily inspection at room temperature is not harmful in our experiments with these copepods.

Our next set of experiments was designed to test the effect of the concentration of food organisms on the survival and growth of *Calanus* at temperatures of 7° or 8° C. For this purpose culture media were prepared in which the concentration of food organisms differed by

TABLE II

Effect of *change* of temperature on survival. Comparison of number of deaths in cultures removed from constant temperature bath for daily periods of varying lengths with number of deaths in cultures not removed. Two hundred and fifty cc. Berkefeld filtered sea water from tap in each culture dish, left unchanged, and no food added. Experiments carried out in July, 1937 at Woods Hole.

Species	Initial no. of animals in each culture dish	Temp. of constant temperature bath	Period culture removed from bath each day	Temp. of culture when replaced	Duration of experiment	No. of deaths
		° C.		° C.	days	
<i>Calanus</i> (Adults)*	10	8.5	30 minutes	14.0	8	1
“ “	10	8.3	not removed		8	3
<i>Calanus</i> (Stage V)†	15	8.0	60 minutes	16-19	7	0
“ “	15	8.0	not removed		7	1
<i>Centropages</i> (Adults)*	20	8.5	30 minutes	12-15	8	17
“ “	22	8.5	120 “	16-21	8	15
“ “	20	8.2	not removed		8	12
“ “	20	13.3	30 minutes	15-18	8	18
“ “	20	13.3	120 “	20-21	8	20
“ “	20	13.0	not removed		8	18

* Animals obtained at Whistle Buoy at western entrance to Vineyard Sound.

† Animals obtained at South Channel, east of Nantucket.

very large amounts (Table III). Ten to twenty-five *Calanus* were introduced into each culture dish and their condition noted each day for the duration of the experiments (2 to 4 weeks). The daily transfer of animals to freshly prepared culture media prevented an appreciable change in the concentration of the food from being brought about by either the grazing of the animals or the multiplication of the food organisms. Accumulation of metabolites was similarly avoided.

In this set of experiments no copepods were observed to moult successfully but certain individuals reached the moulting stage and died during the casting of the shell. As in previous investigations, the

number of animals which began the moulting process may be taken as an index of growth. The reliability of this procedure is strengthened by the fact that low numbers of "deaths in moult" are usually accompanied by high numbers of "deaths not in moult" and vice versa (see Table III). On this basis our results reveal the best nutritional conditions in those cases where 30,000 cells/cc. of *Nitzschia*, or more, were provided, but a concentration as high as a million and a half cells/cc. appeared harmful. The moulting stage was attained (i.e. "deaths in

TABLE III

Effect of concentration of food on survival of *Calanus*. Temperature of constant temperature bath for experiments in July and August: 8° C., in September: 7° C. Each group of animals placed in 250 cc. of culture medium. Animals in copepodid Stage V. Experiments conducted at Woods Hole in 1937.*

Food organism	Conc. of food	Exper. begun	Exper. concluded	Initial no. of copepods	Deaths in moult	Deaths not in moult
	<i>cells/cc.</i>					
Nitzschia	300,000	July 11	Aug. 9	15†	8	5
"	150,000	"	"	15	10	4
"	30,000	"	"	15	8	3
"	15,000	"	"	15	4	8
none		"	"	15	4	8
Nitzschia	1,500,000	Aug. 15	Aug. 31	25‡	0	25
"	150,000	"	Sept. 3	25	1	13
"	1,500	"	"	25	4	16
none		"	"	25	0	11
Unfiltered	harbor water	"	"	25	0	22
Platymonas	18,000	Sept. 11	Sept. 31	10‡	0	7
"	4,500	"	"	10	0	6
Nitzschia	230,000	"	"	10	0	9
none		"	"	10	0	6

* Experiment begun September 11 carried out with the assistance of Mr. Dean F. Bumpus, biological technician for the Woods Hole Oceanographic Institution.

† Animals obtained from South Channel, east of Nantucket.

‡ Animals obtained from Cape Cod Bay, 4 miles NNE of Canal.

moult") in a higher proportion of cases in the tests begun on July 11 than in those begun on August 15 and in the experiment of September 11 not a single copepod reached the moulting condition. Indication that the animals stopped feeding toward the end of the season arises from the fact that no significant difference was observed in the survival in different food concentrations for the September 11 experiment. Moreover, in this case and in the experiment of August 15 the unfed controls fared as well or better than the copepods which were provided with

food. This suggests that the feeding or the survival (or both) of the copepods may depend upon the season because of differences in the phase of the life cycle characterising the copepods at the time the experiments were begun (see below).

With the possibility of a seasonal effect in mind, arrangements were made for the next set of experiments to be conducted during the spring months when *Calanus* off our shores is known to be breeding and grow-

TABLE IV

The effect of temperature and food concentration on the survival and growth of *Calanus*. Animals obtained off Gloucester. Each group placed in 250 cc. of culture medium. Experiments conducted at Harvard Biological Laboratories in 1938 with the assistance of Mr. Dean F. Bumpus.

Jar	Temp.	Conc. of Nitzschia	Initial population April 22. Copepodid stage	Successful moults	Deaths in moult	Deaths not in moult	Final population May 18. Copepodid stage
		cells/cc.					
1	3° C.	150,000	10 IV	5	0	1	1 IV, 7 V†
2		1,500	8 IV, 2 III	4	0	2	2 IV, 6 V
3		0	9 IV, 1 III	5	0	3	2 IV, 4 V†
4	6° C.	150,000	11 IV	10	0	5	3 V, 3 VI♂
5		1,500	10 IV	6	0	5	3 V, 1 VI♂, 1 VI♀
6		0	8 IV, 2 III	0	1	9	0
7	9° C.	150,000	9 IV, 1 III	7	1	4	1 V, 1 VI♂, 2 VI♀†
8		1,500	10 IV	5	1	5	3 V, 1 VI♀
9		0	10 IV	2	1	8	1 V
10*	3° C. then 9° C.	1,500	10 III	4	1	6	2 V†
11	6° C.	1,500	10 III	2	2	7	1 V
12	9° C.	1,500	10 III	6	0	1	?†

* At the end of 7 days at 3° C. no animals had moulted and none had died. The jar was then transferred to 9° C.

† One animal missing.

‡ Animals accidentally lost on the seventh day.

ing (Fish, 1936; Clarke and Zinn, 1937). In nine tests at three temperatures and with varying amounts of food using copepodid stage IV and in three tests using copepodid stage III, run in exactly the same manner as the experiments of the previous summer, survival was good and a considerable amount of moulting took place (Table IV). In the case of stage IV the number of successful moults at 3° C. did not differ materially with the amount of food provided, but at the other

temperatures a distinct positive relation was found between the growth of the animals and the concentration of *Nitzschia*. The largest number of animals moulting occurred in the culture with high food concentration at 6° C. (Jar 4). Deaths during moult took place chiefly at the high temperatures. Deaths not in moult were much more abundant in the cultures not provided with food and were confined largely to the higher temperatures.

Of the stage III animals, all of which were provided with the same amount of food, the group at 9° C. was accidentally lost on the seventh day but six in this group had already moulted. In contrast, there were no signs of growth by the seventh day in the group at 3° C. However, after the latter animals had been transferred to the 9° bath, four moults occurred within six days. In the culture at 6° C. there were two successful moults and two deaths in moult. Three of the stage III animals attained stage V, thus having passed through two moults. In the case of the copepods which were in stage IV at the beginning of the experiment, nine individuals (in the 6° and 9° cultures) moulted twice and thus reached the adult condition (stage VI).⁴ From the results of this set of experiments we may conclude that at 3° C. moulting was retarded but at the same time survival was good. A larger number of successful moults and deaths in moult occurred at 6° C. and about the same proportion at 9° C. but at these temperatures survival was definitely inferior to that at 3° C.

RESPIRATION

Material and Methods

The copepods used for the experiments on respiration were taken by tow net in Cape Cod Bay and placed in large jars packed in ice for the return run to the laboratory. Vigorous specimens of copepodid stage V were then picked out by pipette, transferred to culture dishes and placed in the refrigerator at 8° C. until the tests were begun on the following day. The sea water used for the cultures and for the experiments was taken in carboys at the point where the animals were collected (except for the tests at 16.8° C. in which the laboratory salt water supply was used). In all cases the sea water was Berkefeld filtered and aerated before use. No food was added.

Respiration was measured by the Winkler method and in other tests by a manometric method in which a respirometer of the Dixon-Haldane constant pressure type was used. In the former case almost exactly the same procedure was followed as that described by Marshall, Nicholls, and Orr (1935) except that in the tests reported below 25 and

⁴ Under the microscope the gonads of both sexes were observed to be ripening at the end of the experiment.

75 animals were used respectively in 200 cc. of water instead of the 120 animals employed by the earlier workers. In the latter case the apparatus was arranged as outlined by Dixon (1934) with the exception that no KOH well for the absorption of CO_2 was used. Tests showed that no difference in the changes of value of the gas within the apparatus occurred when the KOH well was removed. It is probable that over the short periods of time involved the small amount of CO_2 produced was readily absorbed by the slightly alkaline sea water. In our apparatus all joints were of ground glass and were carefully sealed with stopcock

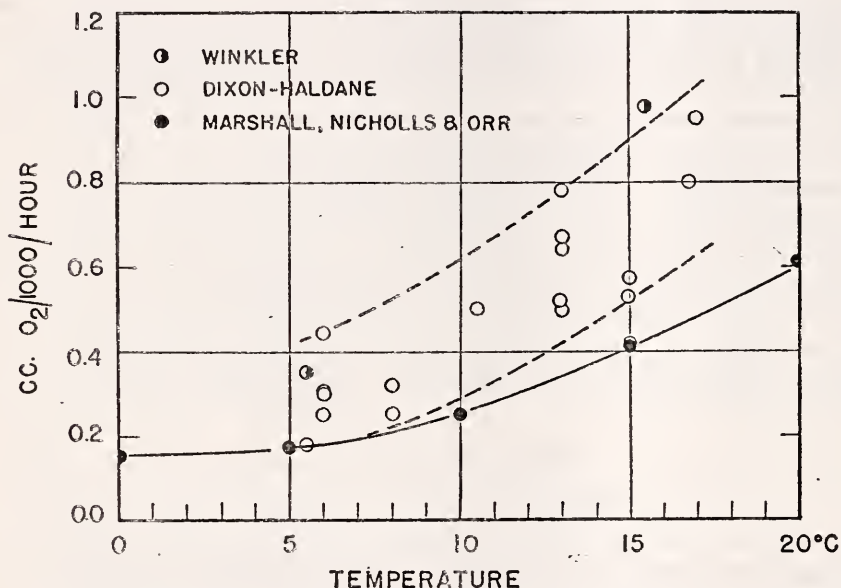


FIG. 1. Relation between temperature and amount of oxygen consumed by stage V *Calanus*. Dashed lines give the limits of all determinations with the Dixon-Haldane respirometer. Experiments carried out at Woods Hole during August and September, 1938.

grease or vaseline. The respiration flasks were completely immersed in a constant temperature bath controlled to 0.5°C .

In the experiments which follow 6 to 30 animals were placed in 10 to 20 cc. of water in one of the respiration flasks. This flask had been painted black to exclude light since Marshall, Nicholls, and Orr reported that differences in illumination exerted a marked influence on the rate of respiration. Equilibrium between the liquid and the gas above it was attained by attaching the entire apparatus to a Warburg shaker. Since continuous or rapid shaking resulted in poor survival of the copepods and might influence abnormally the rate of respiration, the

shaking was limited to the 5 or 10 minutes immediately before each reading and was confined to an amplitude of 2 cm. at a frequency of 100/min. Readings were ordinarily made every 20 minutes for 4 or more hours. The change in volume for each period was observed and reduced to cc. dry gas at N. T. P. Blank trials, which were run at intervals in exactly the same way but with no animals, showed no variation in volume over periods as long as 4 hours. In certain cases water in which copepods had been living and which presumably contained

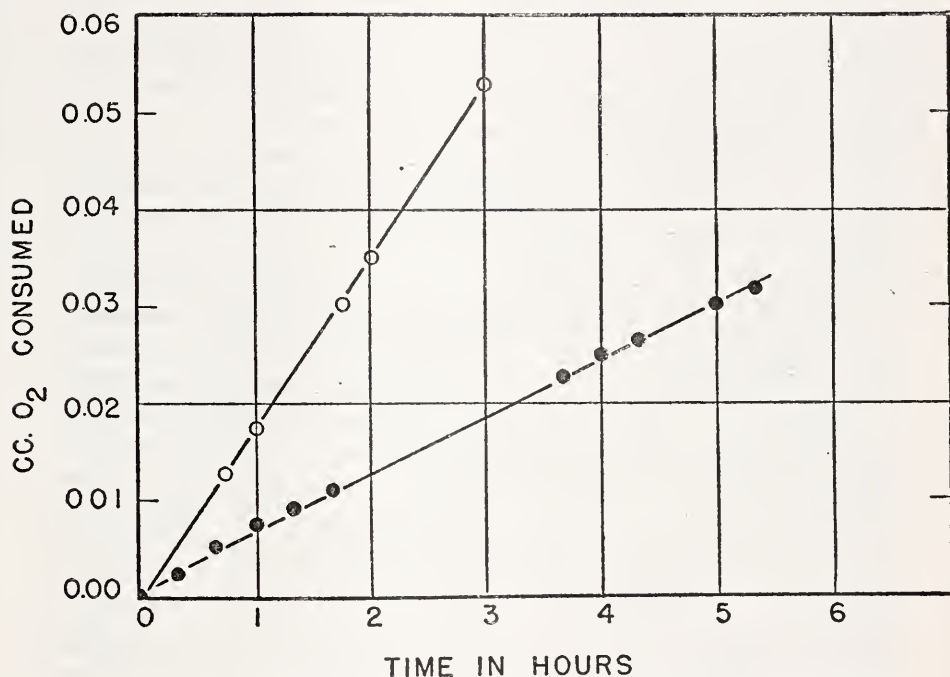


FIG. 2. Progress of two typical experiments on consumption of oxygen by stage V *Calanus*. Closed circles: 6 animals at 16.8° C. Open circles: 28 animals at 13.0° C.

a characteristic number of bacteria was used for the blank tests. Thus assurance was provided that in the experiments neither microorganisms nor other extraneous agents were interfering appreciably with the results.

Experiments

Two measurements of the oxygen consumption of *Calanus* were made using the Winkler method. The first of these was carried out at 5.5° C. using 25 animals confined in 200 cc. of sea water for 4 hours and yielded a value of 0.35 cc. O₂/hr./1,000 copepods. In the second

case 75 animals in 200 cc. were tested at 15.5° C. for 4 hours and were found to have consumed oxygen at the rate of 0.98 cc. O₂/hr./1,000 copepods. The first of the values is somewhat higher and the second is considerably higher than those obtained by Marshall, Nicholls, and Orr (1935), as may be seen in Fig. 1.

The experiments using the Dixon-Haldane respirometer comprised 17 tests carried out at 7 temperatures ranging from 2.5° C. to 16.8° C. In each test from 4 to 12 readings were made during the course of the run and these were plotted against time, as in the two typical experiments shown in Fig. 2. The fact that these curves and all the others which have been used in the calculations exhibited only a slight deviation from a straight line shows that the consumption of oxygen was sensibly constant over the period of each experiment. Marshall, Nicholls, and Orr reported a drop in oxygen consumption during the first 10 or 15 hours after the capture of the copepods but in our experiments, since measurements were not begun until the day following capture, this period of decreasing respiration was avoided.

From the average slope of each curve of oxygen consumption a value was obtained which was converted into cc. O₂/hr./1,000 copepods and the results plotted in Fig. 1. It will be observed that a considerable variation exists among the several tests at the same temperatures. Evidently respiration in *Calanus* can vary between wide limits and this conclusion is borne out by the observations of the earlier investigators. However, there is no doubt as to the general magnitude of the oxygen requirement of *Calanus* in the present experiments. The influence of temperature on respiration is indicated by the upward slope of the dashed lines in Fig. 1 enclosing all the points. The values increased from an average of about 0.32 cc. O₂/hr./1,000 copepods at 5.5° C. to about 0.91 cc. O₂/hr./1,000 copepods at 16.8° C. Although our measurements give a somewhat higher average rate of respiration at all temperatures than was obtained by Marshall, Nicholls, and Orr, they are of the same order of magnitude. Furthermore, there is no indication of a possible lower oxygen requirement for the Woods Hole copepods since *every one* of the present values was higher than those obtained by the British investigators.

DISCUSSION

The foregoing experiments not only have confirmed and illuminated the early work at Woods Hole, but in addition have raised a number of new questions. It is clear that both the amount of food and the temperature have an important influence on the growth of *Calanus* and on

its survival.⁵ The situation is further complicated by the fact that under conditions which tend to promote growth, survival may be poor because of the likelihood of death during the moulting process. The provision of food improves growth and survival, but the exact amount of food seems not as important as originally supposed (cf. Clarke, 1939). Although a minimum was ordinarily required for growth, in certain experiments at low temperatures some moulting was observed in the entire absence of food. And in at least one case a very high concentration of food was found to be harmful (see also Lucas, 1936).

It is a striking fact that a large amount of moulting took place and good survival was observed in our experiments conducted in April and May (Table IV) whereas poor survival and no successful moulting was the rule for the tests made during the summer and early autumn (Table III) and for the summer experiments of Fuller (1937, Table V, temperature 13° C.).⁶ In all cases *Nitzschia* was used as the food organism in at least some of the cultures, the temperatures of the cultures were not widely different, and exactly the same procedure was followed. For the April and May tests copepods were obtained from a point off Gloucester but in two of the other cases the experimental animals were procured from a location which was also north of Cape Cod although considerably to the south of Gloucester.⁷ If the place of origin of the copepods is immaterial, it might be suggested that the time of year at which the animals were collected could have an important bearing on the laboratory results. According to the observations of Clarke and Zinn (1937) and of Fish (1936) the second of the two yearly broods of *Calanus* grows to copepodid stage V during the summer and remains in that stage until the following January or February. It seems possible that in nature feeding may be reduced to a minimum and mortality may be especially high during the late summer and that animals collected during this period might exhibit poor survival and refuse to grow even under the most favorable laboratory conditions. Added support for this view is derived from the fact that in our experiment begun September 11 (Table III) no successful moults occurred and no animals were observed even to attempt to moult.

⁵ In fact Fish (1937) is of the opinion that the presence of suitable temperatures is the most important factor in the production of zoöplankton in the Gulf of Maine and the Bay of Fundy.

⁶ In the earlier summer experiments of Clarke and Gellis (1935) and of Fuller and Clarke (1936) growth and survival were better, but the different methods used make comparisons inappropriate.

⁷ The following ocean temperatures were observed:

Off Gloucester, April 26, 1938, 9.6° C. at surface.

Cape Cod Bay, August 14, 1937, 18.3° C. at surface, 10.2° C. at 15 m., 8.6° C. at 30 m. South of Cape Cod temperatures were generally higher.

In all of our experiments at Woods Hole and especially in those described above the copepods were noticed to be heavily parasitized immediately after death. An animal which appeared perfectly healthy one day would be found dead on the next day with its body filled with opaque whitish or colored masses. Possibly parasites gain a foothold within the copepod and remain unnoticed until they suddenly reach some vital tissue. From this initial infection the rapid and obvious spread of the parasite to all parts of the body could be accomplished after death within a relatively short interval. Since Jepps (1937) reported that a large proportion of *Calanus* population was found to be infected when collected, it is possible that the inroads of parasites may be an important element underlying the fluctuation in the abundance of this species both seasonally and annually.

The experiments on respiration show that the values which have been used for the oxygen consumption of *Calanus* and hence its food requirement, are of the correct order of magnitude. In fact, our measurements indicate that an even larger amount of food per day is required by these organisms than was assumed by Marshall, Nicholls, and Orr. Therefore, the discrepancy between the nutrient required and the number of diatoms available for food gapes wider than ever. According to Harvey (1937) *Calanus* can filter out the larger species of diatoms much more effectively than the smaller forms. He found that 100 times the volume of water may be swept free in an hour when *Lauderia* or *Ditylium* were provided as food as when *Nitzschia* was used. However, in nature the larger diatoms are not as abundant as the more minute species, and a careful investigation of the amount of food per cc. of sea water which the scarcer but more bulky types would provide is seriously needed. Another point which needs study is the "micro-distribution" of the phytoplankton and nannoplankton. Although uniformity in abundance may be found in a series of one-liter water samples taken from an area, the distribution *within* each liter may be highly irregular. If the food organisms occur in clusters, and if a copepod can find and stay in a local though minute zone of abundance, sufficient nutriment could be obtained without the necessity of filtering an excessive volume of water. Such explanations as the foregoing may resolve the discrepancy between the daily food requirement and the *average* abundance of potential food organisms.

SUMMARY

Laboratory tests on cultures of *Calanus* in relation to the ecology of this species showed that: (a) removal of the culture dishes from constant low temperature to room temperature for daily periods as great

as 120 minutes was not harmful to the copepods; (b) growth was poorer at 3° C. than at 6 to 9° C. but survival is better at the lower temperatures; (c) both growth and survival decreased regularly in passing from experiments conducted in the spring to those in the autumn.

Measurements of the respiration of *Calanus* using the Winkler method and the Dixon-Haldane respirometer showed that the magnitude of the oxygen requirement for our animals is of the same order as for those tested by Marshall, Nicholls, and Orr, and possibly is higher than previously reported. The discrepancy between the estimated food requirement of *Calanus* and the average abundance of diatoms therefore still exists but certain possible explanations are discussed.

REFERENCES

- CLARKE, G. L., 1939. The relation between diatoms and copepods as a factor in the productivity of the sea. *Quart. Rev. Biol.*, in press.
- CLARKE, G. L., AND S. S. GELLIS, 1935. The nutrition of copepods in relation to the food-cycle of the sea. *Biol. Bull.*, 68: 231-246.
- CLARKE, G. L., AND D. J. ZINN, 1937. Seasonal production of zoöplankton off Woods Hole with special reference to *Calanus finmarchicus*. *Biol. Bull.*, 73: 464-487.
- DIXON, MALCOLM, 1934. *Manometric Methods*. Cambridge University Press.
- FISH, C. J., 1936. The biology of *Calanus finmarchicus* in the Gulf of Maine and Bay of Fundy. *Biol. Bull.*, 70: 118-141.
- FISH, C. J., AND M. W. JOHNSON, 1937. The biology of the zoöplankton population in the Bay of Fundy and Gulf of Maine with special reference to production and distribution. *Jour. Biol. Board of Canada*, 3: 189-321.
- FULLER, J. L., 1937. Feeding rate of *Calanus finmarchicus* in relation to environmental conditions. *Biol. Bull.*, 72: 233-246.
- FULLER, J. L., AND G. L. CLARKE, 1936. Further experiments on the feeding of *Calanus finmarchicus*. *Biol. Bull.*, 70: 308-320.
- GROSS, F., 1937. Notes on the culture of some marine plankton organisms. *Jour. Mar. Biol. Ass'n*, 21: 753-768.
- HARVEY, H. W., 1937. Note on selective feeding by *Calanus*. *Jour. Mar. Biol. Ass'n*, 22: 97-100.
- JEPPS, M. W., 1937. On the protozoan parasites of *Calanus finmarchicus* in the Clyde Sea area. *Quart. Jour. Micros. Sci.*, 79: 589-658.
- LUCAS, C. E., 1936. On certain inter-relations between phytoplankton and zoöplankton under experimental conditions. *Jour. du Conseil Int. pour l'explor. de la mer*, 11: 343-362.
- MARSHALL, S. M., A. G. NICHOLLS, AND A. P. ORR, 1935. On the biology of *Calanus finmarchicus* Part VI. Oxygen consumption in relation to environmental conditions. *Jour. Mar. Biol. Ass'n*, 20: 1-28.

DEVELOPMENT OF HALF-EGGS OF CHAETOPTERUS PERGAMENTACEUS WITH SPECIAL REFERENCE TO PARTHENOGENETIC MEROGONY

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It has been shown in previous studies on sea urchin eggs (1936, 1938) that the non-nucleate fractions, obtained by centrifugal force, may be activated with parthenogenetic agents and may cleave and develop, without nuclei, to the blastula stage. This I have termed parthenogenetic merogony. Five species of sea urchins have given similar results. I had hoped that the parthenogenetic merogone of another class of animal might develop further than the blastula, and for this reason have made the present study.

The egg of the worm, *Chaetopterus*, fulfills the two requirements necessary for parthenogenetic merogony. First, it can be broken apart readily by centrifugal force so that one can obtain non-nucleate fractions in abundance, and second, the normal egg can easily be made to develop parthenogenetically by treatment with salts. Although my chief concern has been the development of the parthenogenetic merogones, a study has also been made of the fertilized merogones for comparison, as well as of the whole centrifuged egg and the white (nucleate) halves both fertilized and parthenogenetic. Although some of my observations may seem to repeat the earlier ones of Lillie (1906, 1909) and Wilson (1929, 1930), the present study is on fractions of a definite size and known nuclear and cytoplasmic composition whereas previous observations have been made on fragments of irregular size and composition and of assumed nuclear content. Certain differences in my results as compared with previous work may be attributed to this difference in material. Other discrepancies are due to the greater centrifugal force used in these experiments, but particularly to the fact that the eggs have been centrifuged in a medium of cane sugar and sea water. The sugar changes the surface of the egg, and in fact sometimes acts on these eggs as a parthenogenetic agent, similar to KCl. A sugar solution was used as a medium because it can be made, by the addition of the proper amount of sea water, of the same specific gravity as the eggs (as well as, by proper dilution, of the same osmotic pressure), so that the eggs remain suspended during centrifugation, and are free to break into two

halves. It also permits the eggs to orient with the lighter area, containing the polar spindle, toward the centripetal pole of the centrifuge, whereas in sea water alone the heavy eggs are quickly thrown to the bottom of the centrifuge tube and are held in the position in which they are thrown. Although no special study has been made of the relation of polarity of the egg and the cleavage planes to the stratification and plane of breaking caused by the centrifugal force, there seems no doubt from observations with the centrifuge microscope that the eggs in the sugar solution *do* orient with the polar area toward the centripetal axis of the centrifuge. It may be mentioned in passing that the *Arbacia* egg in the 12-cell stage, when the colorless micromeres appear, also orients with the micromeres toward the centripetal pole of the centrifuge; this can be readily observed with the centrifuge microscope. The lack of pigment granules makes these cells lighter than the other cells, which are pigmented. In the *Chaetopterus* egg, the clear polar region of the egg when ready for fertilization (Photograph 18) is lighter than the granular region and therefore the egg orients as stated above. When the eggs are centrifuged immediately on laying, in the germinal vesicle stage (Photographs, 17, 3), there is no visible lighter area, and there is no apparent orientation. This is, I think, in line with Morgan's studies (1937, 1938); he has definitely determined that the germinal vesicle is not eccentric even in eggs still in the parapodia; it therefore cannot cause orientation.

Stratification and Breaking

(Photographs 1-16)

The *Chaetopterus* egg stratifies readily. In fact, simply leaving the eggs undisturbed over night in isotonic KCl produces a very nice stratification (Photograph 1). This is true also for the *Arbacia* egg to a slight extent. The *Chaetopterus* egg, both unfertilized and fertilized, stratifies also readily with centrifugal force (Photograph 2). It stratifies similarly to the *Arbacia* egg except that there is no pigment; the stratified egg is almost exactly like that of another sea urchin, *Sphaerechinus granularis*. Some photographs of the stratified eggs of the annelid, *Chaetopterus*, and of the echinid, *Spaerechinus*, are very similar and might easily be mistaken the one for the other except that there is a little more oil in the *Chaetopterus* egg (Cf. Photographs 4 and 5). The unfertilized eggs of *Chaetopterus* were usually in these experiments centrifuged after the polar spindle had formed; it was found difficult to stratify them well (Photograph 3) and almost impossible, with the forces used, to break them apart in the very short interval elapsing

between laying and the breaking of the germinal vesicle. The oil goes to the light pole, then there is a clear layer, then a band of mitochondria which stains purple with methyl green, and then the yellow yolk granules at the heavy pole. (Photographs 4, 6). The nucleus, or rather the first polar spindle, *always* lies in the clear zone under the oil. The eggs are very heavy and are usually thrown to the bottom of the centrifuge tube in the same sugar solution in which *Arbacia* eggs are nicely suspended. The result is that the egg breaks irregularly; usually a small oil cap is thrown off and the rest of the egg breaks into irregular fragments (Photographs 13, 14 of eggs in the centrifuge microscope slide). This is also the case when the eggs are centrifuged in sea water alone. The earlier experiments of Lillie and Wilson and most of Morgan's experiments were done in this way.

It is possible, however, to keep the eggs suspended during centrifugation by using a minimum of sea water with the eggs, and a large amount of the isotonic sugar solution. In this way, after centrifuging

PLATE I

Stratification and Breaking of Eggs

The photographs were all taken of living eggs with a Leica camera, and for the most part with a water immersion ($\times 40$) lens. The magnification on the plates with the exception of Plate II is approximately $175\times$; Plate II is approximately $250\times$. The smaller photographs were taken with a low power ($\times 10$) lens and are here magnified about $65\times$. The times (i.e., minutes, hours) given are derived from the data recorded at the time of photographing.

PHOTOGRAPH 1. *Chaetopterus* egg kept overnight undisturbed in isotonic KCl.

PHOTOGRAPH 2. Egg centrifuged (after fertilization) for comparison with Photograph 1.

PHOTOGRAPH 3. Egg centrifuged immediately on laying; shows unbroken germinal vesicle; this broke immediately afterwards.

PHOTOGRAPH 4. Stratified *Chaetopterus* egg; oil, clear layer, mitochondria and yolk. Polar spindle is in the clear layer under the oil.

PHOTOGRAPH 5. Stratified egg of *Sphaerechinus granularis* for comparison with Photograph 4; same layers.

PHOTOGRAPHS 6-9. Breaking apart of stratified egg of *Chaetopterus* into two halves of uniform size.

PHOTOGRAPH 10. White half-eggs, containing oil, clear layer, mitochondria and a little yolk; also polar spindle. Note uniformity of size.

PHOTOGRAPH 11. White half-egg showing band of mitochondria; stained with methyl green.

PHOTOGRAPH 12. Yellow half-egg, containing yolk.

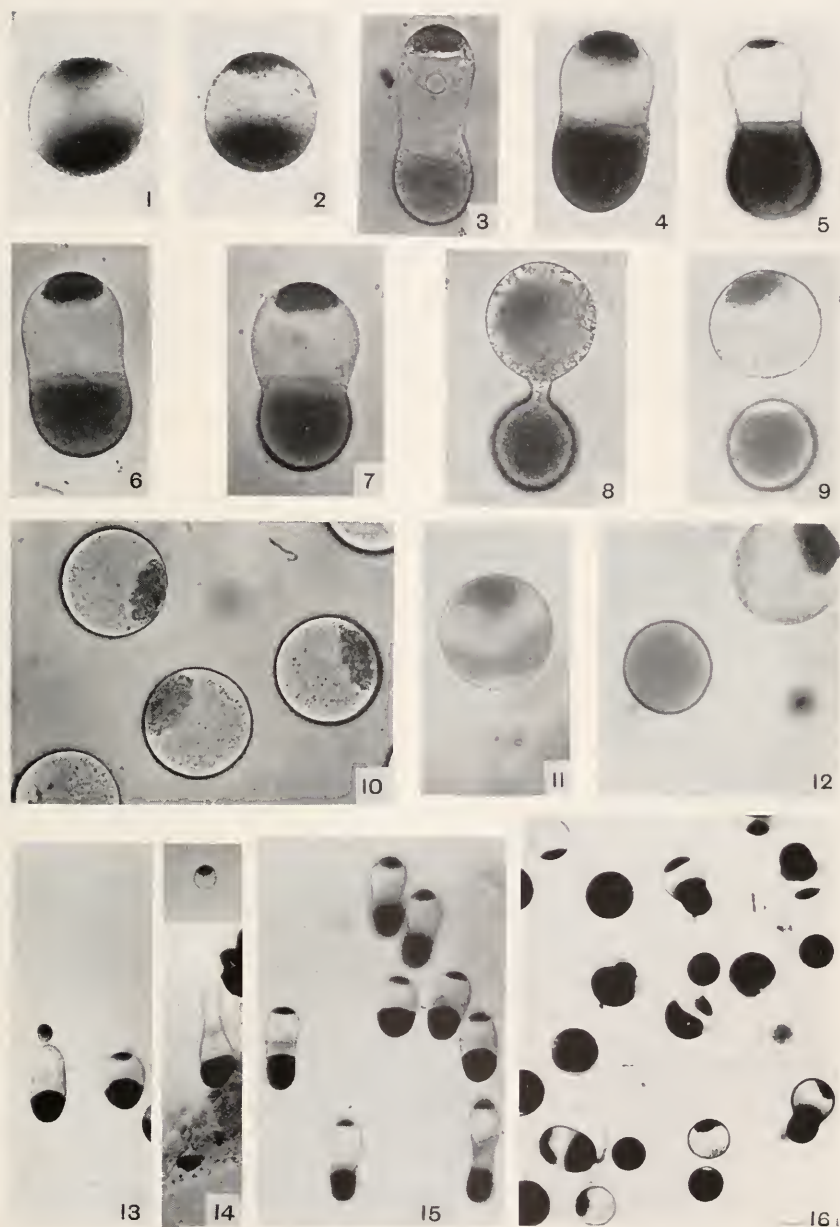
PHOTOGRAPHS 13-15. Eggs immediately after removal from the centrifuge microscope, and still in the centrifuge microscope slide.

PHOTOGRAPHS 13, 14. Eggs supported at bottom of slide. Oil cap pulls off and egg breaks irregularly.

PHOTOGRAPH 15. Eggs suspended in sugar solution. Ready to break into halves of uniform size.

PHOTOGRAPH 16. A group of eggs just removed from the centrifuge tubes, showing regularity of breaking and uniformity of size of halves. The larger black spheres are the whole eggs viewed from above.

PLATE I



Photographs 1-16

for five minutes at $10,000 \times$ g., the eggs stratify, become elongate, then dumbbell shape, and then break into spheres, quite regular in size and quite similar to the halves of the *Arbacia* egg (Photographs 6-9, 16; 15 in the centrifuge microscope slide). The *Chaetopterus* egg breaks, so that the lighter or white half contains oil, clear layer, nucleus (or polar spindle), mitochondria and a little yolk (Photographs 9, 10, 11). The heavier yellow half contains only yolk (Photographs 9, 12). The measurements for the whole and half eggs are:

Whole egg	Diameter	94 μ	Volume	434900 μ^3
White half	"	83 μ	"	299400 μ^3
Yellow half	"	64 μ	"	137300 μ^3
				436700 μ^3

The white half has over twice the volume of the yellow half. Many batches of eggs have given similar figures.

Normal Development

(Photographs 17-31)

As is well known, especially from the early work of Wilson (1883) and Mead (1895, 1897, 1898 *a, b*), the early development of the egg is as follows. The egg is laid in the germinal vesicle stage (Photograph 17); after resting in sea water a few minutes, the germinal vesicle

PLATE II

Normal Development of the Chaetopterus Egg

PHOTOGRAPH 17. Immature egg immediately on laying; germinal vesicle still intact in center.

PHOTOGRAPH 18. Egg 15 minutes after laying; polar spindle in white area at upper pole.

PHOTOGRAPH 19. Fertilized egg, showing crinkled or fluted fertilization membrane. Twenty-five minutes after fertilization.

PHOTOGRAPH 20. Egg flattened where first polar body is being extruded. Fifteen minutes after fertilization.

PHOTOGRAPH 21. Egg rounded again after first polar body has been extruded; note fluted membrane near polar body. Twenty-five minutes after fertilization.

PHOTOGRAPH 22. Egg flattened again at time of formation of second polar body. Thirty minutes after fertilization.

PHOTOGRAPH 23. Same egg 1 minute later; it has again rounded out.

PHOTOGRAPH 24. Pear-shaped stage. Thirty-four minutes after fertilization.

PHOTOGRAPH 25. Same egg 2 minutes later; beginning of polar lobe.

PHOTOGRAPH 26. Same egg 4 minutes later; 40 minutes after fertilization.

First cleavage beginning, with polar lobe still present.

PHOTOGRAPH 27. Same egg 20 minutes later; first cleavage complete and polar lobe withdrawn; note polar bodies near furrow at upper pole.

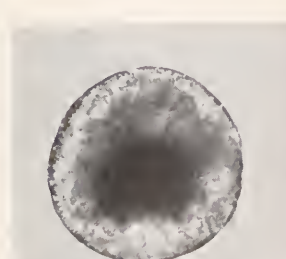
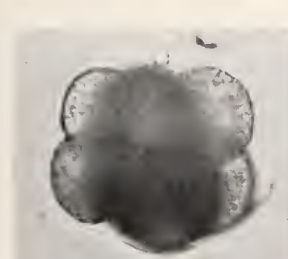
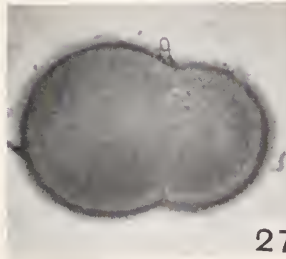
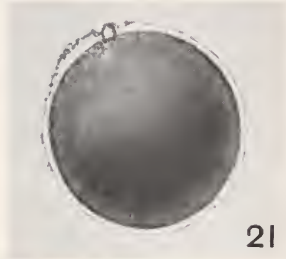
PHOTOGRAPH 28. Four-cell stage, 1 hour, 40 minutes after fertilization. Note characteristic *Brechungsline* or cross-furrow.

PHOTOGRAPH 29. Eight-cell stage, 2 hours after fertilization.

PHOTOGRAPH 30. Blastula, just before swimming; $4\frac{1}{2}$ hours after fertilization.

PHOTOGRAPH 31. Trochophore, 1 day after fertilization.

PLATE II



breaks and the first polar spindle forms. The polar region is seen in the living egg as a clear area near the surface; it is fully formed (at 23° C.) 10–20 minutes after laying (Photograph 18). The egg remains in this condition, with the first polar spindle in the metaphase, indefinitely unless fertilized. If fertilized at this stage, in about 15 minutes¹ the first polar body appears (Photograph 20). The fertilization membrane is the same, according to Whitaker (1933), as the vitelline membrane which lifts from the surface. It is peculiar and characteristic for this egg and quite different from the fertilization membrane of sea urchin eggs. It is crinkled or fluted especially in the region of the polar bodies (Photographs 19, 21). The egg becomes markedly flattened at the pole at the formation of the first polar body (Photograph 20); then becomes spherical (Photograph 21). Then the second polar body is formed, again with a slight flattening (Photograph 22)—about 30 minutes after fertilization; and the egg once again rounds out (Photograph 23). Soon after this (35 minutes after fertilization) the egg becomes pointed or pear-shaped with the narrow end toward the polar bodies (Photograph 24). After again rounding out, the characteristic polar lobe is formed (about 5 minutes after the pear-shape) at the opposite end (Photograph 25). This remains till after first cleavage which begins about 40 minutes after fertilization (Photograph 26). The first cleavage is very unequal giving a small cell and a large one into which the yolk lobe is absorbed (Photograph 27). At the second cleavage, 1½ hours after fertilization, the small cell divides equally and the large cell unequally so that there are two small cells, one large cell and one intermediate in size (Photograph 28), a form of cleavage quite typical for many annelid and mollusk eggs. The next cleavage is shown in Photograph 29, taken 2 hours after fertilization. The spiral type of cleavage which occurs in *Chaetopterus* is difficult to follow in the living egg, so that no attempt has been made to photograph the later cleavage stages of the normal egg, nor to follow them in the experimental work. A normal blastula is shown in Photograph 30, taken just before swimming, which occurs about 5 hours after fertilization; and a one-day trochophore, in Photograph 31.

¹ The times for the various stages are quite variable in the *Chaetopterus* egg even with constant temperature. It differs in this respect from the *Arbacia* egg in which, in the height of the season, the stages occur with almost clock-like regularity at any given temperature. Late in the season (September, October) there is considerable delay in the *Arbacia* egg irrespective of temperature. Eggs may be obtained through November from *Arbacias* which have been brought in before the first part of August and kept in the aquaria in running water. Animals brought in from the sea after August 15 have practically all shed their eggs.

Normal Egg, Parthenogenetic

The parthenogenetic development of the normal uncentrifuged egg by the addition of KCl to the sea water has been described by Mead (1898 b), Loeb (1901) and Lillie (1902, 1906), and in detail by Allyn (1912). The peculiar feature is the amoeboid form of the egg and the frequent occurrence of very large nuclei without cell division. The later embryos tend to stick together and form complex multiple organisms as described particularly by Loeb (1901). This is probably due to the effect of the KCl solution on the surface of the cells. Unequal two- and four-cell stages of the characteristic annelid type also occur, and the parthenogenetic egg may give rise to apparently normal swimmers just like the fertilized egg. Some equal two-cell stages also occur.

My best results for parthenogenesis have been obtained by putting the eggs into a solution of 1 gram KCl + 100 cc. of sea water for 30 minutes, then transferring them to sea water. However, I have found other proportions and times of exposure to work better with some batches of eggs. The different batches respond quite differently to the KCl solutions and I could get no dependable standard solution.

Whole Egg Centrifuged, Then Fertilized

(Photographs 32-42)

The *centrifuged* whole egg, which has been elongated by the centrifugal force, becomes spherical again quite quickly after the force is removed, and this whether the egg is fertilized or not. It is thus strikingly different from the sea urchin egg which becomes spherical if left unfertilized, but retains its elongate or dumb-bell shape if fertilized or artificially activated, all through the cleavages up to the swimming blastula. After fertilization of the centrifuged egg, in *Chaetopterus*, the polar bodies usually come off near, but not on top of, the oil cap (Photograph 32); if the oil cap has been centrifuged off, however, they usually come off exactly at the centripetal pole (Photograph 33); the oil cap apparently interferes with the protrusion of the polar bodies. This is shown also by the results of centrifuging the egg immediately on laying, while the germinal vesicle is still intact, as shown in Photograph 3. When, after removal from the centrifuge, the germinal vesicle breaks, it dissipates the oil in clumps (Photograph 34). The polar spindle forces itself to the center of the oil, which now forms a circle of clumps around it (Photograph 35). After fertilization, the polar bodies come off in the center of the ring of oil clumps.

The fertilized centrifuged egg usually passes through the changes in shape characteristic of the normal fertilized egg as described above, in-

cluding the formation of the polar lobe. It often divides unequally and typically in the first cleavage (Photograph 36), and the second cleavage as well as later ones (Photograph 39) may be of the characteristic annelid type as has been described by Lillie (1906) and Wilson (1929). Some of the cleavages, however, especially in strongly centrifuged eggs, are irregular and atypical. The first division plane is usually perpendicular to the stratification (Photograph 36). It may, however, pass parallel with the stratification, separating the egg into a clear and a granular zone (Photograph 37). These zones may develop separately just as in the centrifuged *Arbacia* egg (Harvey, 1932); the upper clear area usually divides into cells but the lower yolk half is characterized by nuclear without cytoplasmic divisions (Photograph 38). Many swimming, apparently normal, larvae have arisen from the fertilized centrifuged eggs (Photograph 40). But the blastulae tend to gather together in groups and fuse with each other and with the white halves,

PLATE III

PHOTOGRAPHS 32-42. *Eggs centrifuged, then fertilized.* Photographs 43-46. *Eggs centrifuged then treated with KCl; parthenogenetic.*

PHOTOGRAPH 32. Polar bodies at side of oil cap; 23 minutes after fertilization.

PHOTOGRAPH 33. Polar bodies at centripetal pole where oil cap has been centrifuged off. Twenty-five minutes after fertilization.

PHOTOGRAPH 34. Egg was centrifuged immediately on laying (see Photograph 3); germinal vesicle breaks after removal from centrifuge and scatters oil in clumps.

PHOTOGRAPH 35. On formation of polar spindle a few minutes later, the clumps of oil are forced into a ring.

PHOTOGRAPH 36. Normal cleavage of centrifuged egg; cleavage plane at right angles to stratification. Note polar lobe. Fifty minutes after fertilization.

PHOTOGRAPH 37. Cleavage plane parallel with stratification. One and one-quarter hours after fertilization.

PHOTOGRAPH 38. Later stage of Photograph 37; upper cells have cleaved several times, lower cell has not cleaved, but the nuclei have divided. One and three-quarter hours after fertilization.

PHOTOGRAPH 39. Normal development of centrifuged egg. Two and one-half hours after fertilization.

PHOTOGRAPH 40. Same, four and three-quarter hours after fertilization; just before swimming.

PHOTOGRAPH 41. Fusion of whole centrifuged and white half-blastulae. Seven hours after fertilization.

PHOTOGRAPH 42. A pair of these, high power.

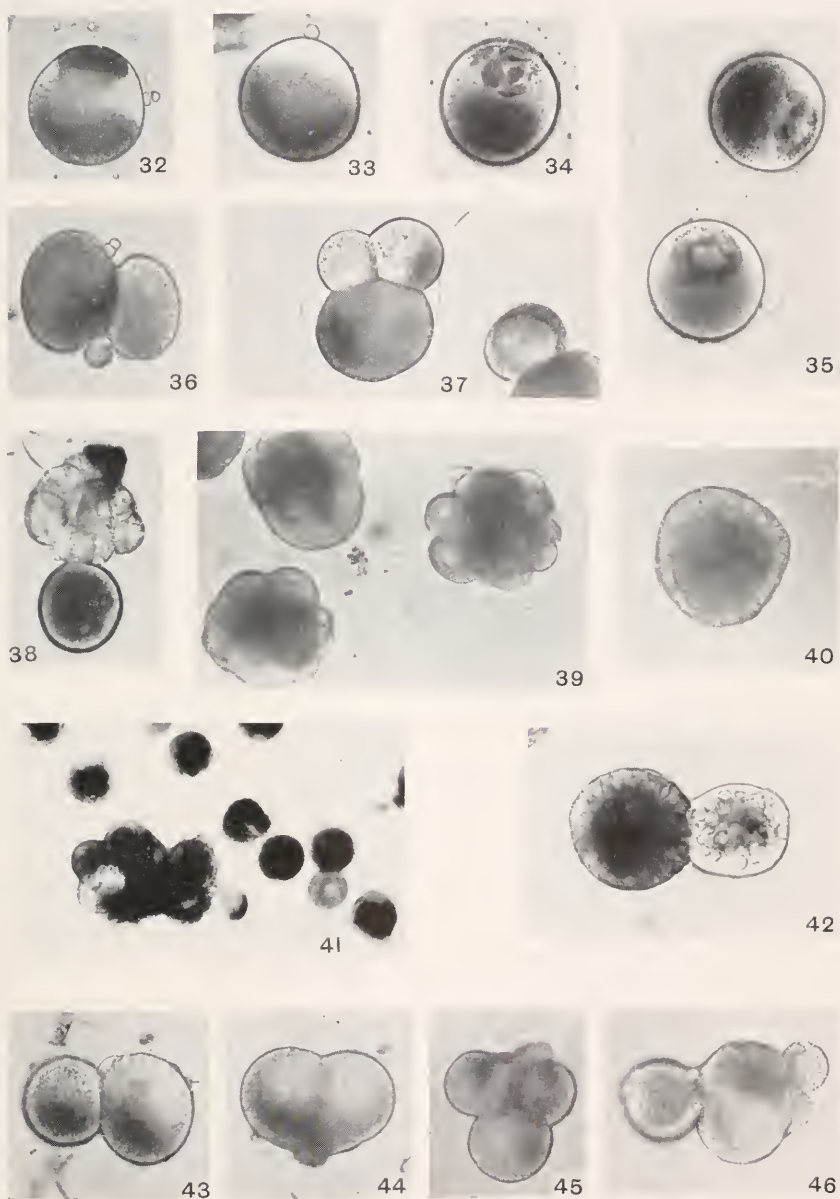
PHOTOGRAPH 43. Two-cell stage of parthenogenetic centrifuged egg; typical unequal blastomeres; cleavage plane perpendicular to stratification. Two hours after treatment.

PHOTOGRAPH 44. Same, equal cells. Note polar lobe.

PHOTOGRAPH 45. Four-cell stage, parthenogenetic. Three hours after treatment. Compare with Photograph 74, of the yellow half-egg fertilized.

PHOTOGRAPH 46. Amoeboid form of parthenogenetic centrifuged egg, 5 hours after treatment. Compare with parthenogenetic white half, Photograph 58; with fertilized yellow half, Photograph 75; and with parthenogenetic merogone, Photograph 96.

PLATE III



Photographs 32-46

forming complex organisms (Photographs 41, 42) just as is characteristic of the parthenogenetic normal eggs. This agglutination must be due to the effect of the sugar solution (in which the eggs have been centrifuged) on the surface of the eggs. It cannot be due entirely to the lack of a fertilization membrane, since eggs from which fertilization membranes have been removed by shaking, and which were kept in sea water, without sugar, do *not* agglutinate.

Centrifuged Egg, Parthenogenetic

(Photographs 43-46)

The centrifuged egg will develop parthenogenetically by the addition of KCl to the sea water, in exactly the same way as the uncentrifuged. Polar bodies may be given off often while still in the KCl solution, and there occur some typical normal cleavages (Photograph 43). But there is a tendency toward equal first cleavage (Photograph 44) and most of the eggs soon become amoeboid and develop with large nuclei and few cell divisions like the uncentrifuged parthenogenetic eggs (Photographs 45, 46). The blastulae tend to fuse together just as do the parthenogenetic normal eggs and the fertilized centrifuged eggs. Development is slower in the parthenogenetic eggs, and swimmers occur in 8 hours instead of 5. This has been found true also of parthenogenetic normal eggs of *Chaetopterus* as well as of many other eggs.

White Half-egg, Fertilized

(Photographs 47-55)

The white half-egg, which contains the polar spindle, can be fertilized, and may develop exactly like the normal whole egg. The egg passes through the flattened and pear-shape phases and the polar bodies are usually given off near the oil cap; these are usually larger than in the normal egg (Photograph 47). The egg usually divides unequally in the first cleavage (Photograph 48, lower egg) and the large cell unequally in the second cleavage giving the typical four-cell stage of two small cells, one large and one intermediate in size (Photograph 49, lower egg). The nuclei are especially striking owing to the lack of granules; these obscure the nuclei in the living whole eggs. There is a tendency for the egg to become amoeboid even before the first division, so that it is not possible to determine if there is a polar lobe or not (Photograph 50). There are often protuberances from one of the blastomeres resembling polar lobes and these occur also in the four-cell stage (Photograph 51). There is also a tendency toward equal first cleavage. Normal blastulae and swimming trochophores are often

formed exactly like those of the whole egg, except that they are white instead of yellow and are smaller (Photograph 52, inset, upper left). But frequently, the blastulae have irregular sized cells and large nuclei like the parthenogenetic whole eggs and they tend to become amoeboid (Photograph 52). The blastula shown in Photograph 53 was both amoeboid and ciliate. These white blastulae often stick together in clumps and fuse with each other and with the whole blastulae present in the same lot (Photographs 54, 55, 41, 42). As pointed out above, this is probably due to the effect of the sugar solution on the surface. The development of the fertilized white halves takes place at about the same rate as the whole eggs; swimmers occur in 5 hours.

The striking thing is that the white half-eggs often cleave, at least in the early stages, exactly like the whole eggs in the very peculiar and characteristic pattern typical of the annelid egg. This has been found also to be the case in many irregular fragments studied by Lillie (1909) and Wilson (1929).

White Half-egg, Parthenogenetic

(Photographs 56-62)

The white half-eggs may be activated by KCl similarly to the whole eggs; some have been activated and have produced swimmers without further treatment than the sugar solution in which they were centrifuged. They may throw off polar bodies often while still in the KCl solution (Photograph 56) and may cleave in the typical annelid fashion (Photograph 57), at least to the four-cell stage. But they, like the whole parthenogenetic eggs, often divide equally at first cleavage, and they usually become amoeboid and develop with irregular cells and large nuclei (Photographs 58-62); these irregularities are much more pronounced in the parthenogenetic than in the fertilized white halves. Development is also delayed as is usual with parthenogenetic eggs.

Yellow Half-egg, Fertilized (Fertilized Merogone)

(Photographs 63-84)

The yellow half-egg, which contains no nucleus, may be fertilized and the fertilization membrane is lifted off. I have observed no polar bodies of which I could be sure, though there are often clear amoeboid protrusions simulating polar bodies. One would not, of course, expect polar bodies in this half-egg, since the polar spindle is always in the other half. There is also no polar lobe of which one can be sure. There is usually no cleavage of this half-egg, but it becomes amoeboid and may live in this condition for several days. In some eggs, I have

found, several hours after fertilization, a nucleus, from the sperm, and later two nuclei, and much later several nuclei of different sizes—that is, nuclear division without cell division (Photographs 63–65), such as is characteristic of the heavy red halves of the *Arbacia* egg (Harvey, 1932). However, several eggs have given a typical first cleavage of two unequal cells (Photographs 66, 67). Several eggs have divided into two equal cells, and in one case these divided again equally giving four equal cells—not typical for this form (Photographs 68, 69). These four cells, later on, fused, cleaved again, became amoeboid and soon went to pieces (Photographs 70–75). In general a failure to cleave and a tendency to become amoeboid are characteristic of the fertilized yellow halves or fertilized merogones. No blastulae or ciliated structures from the fertilized yellow halves have been observed. A series of photographs (76–84) will show the extreme amoeboid activity of one of these eggs in the two-cell stage. These photographs

PLATE IV

White half-eggs, fertilized (47–55) and parthenogenetic (56–62)

PHOTOGRAPH 47. White half fertilized, with polar bodies. Twenty-five minutes after fertilization.

PHOTOGRAPH 48 (lower egg). Typical two-cell stage. One and one-quarter hours after fertilization.

PHOTOGRAPH 49 (lower egg). Typical four-cell stage. One and one-half hours after fertilization. Note *Brechungsline* or cross-furrow as in Photograph 28. In upper egg, all the oil is in one blastomere, and the blastomeres are not quite typical in size.

PHOTOGRAPH 50. Just before first cleavage, amoeboid at position for polar lobe. Fifty minutes after fertilization.

PHOTOGRAPH 51. Four-cell, amoeboid. One hour after fertilization.

PHOTOGRAPH 52. Amoeboid blastulae with large irregular nuclei, and also normal white blastula (inset, upper left). At the upper right is a normal whole blastula (centrifuged). Five hours after fertilization.

PHOTOGRAPH 53. White blastula, amoeboid and also ciliated. Six and one-half hours after fertilization.

PHOTOGRAPH 54. White blastulae fused together. See also Photograph 41. Six hours after fertilization.

PHOTOGRAPH 55. A pair of fused blastulae, high power. See also Photograph 42.

PHOTOGRAPH 56. Parthenogenetic white half with two polar bodies. One hour after activation.

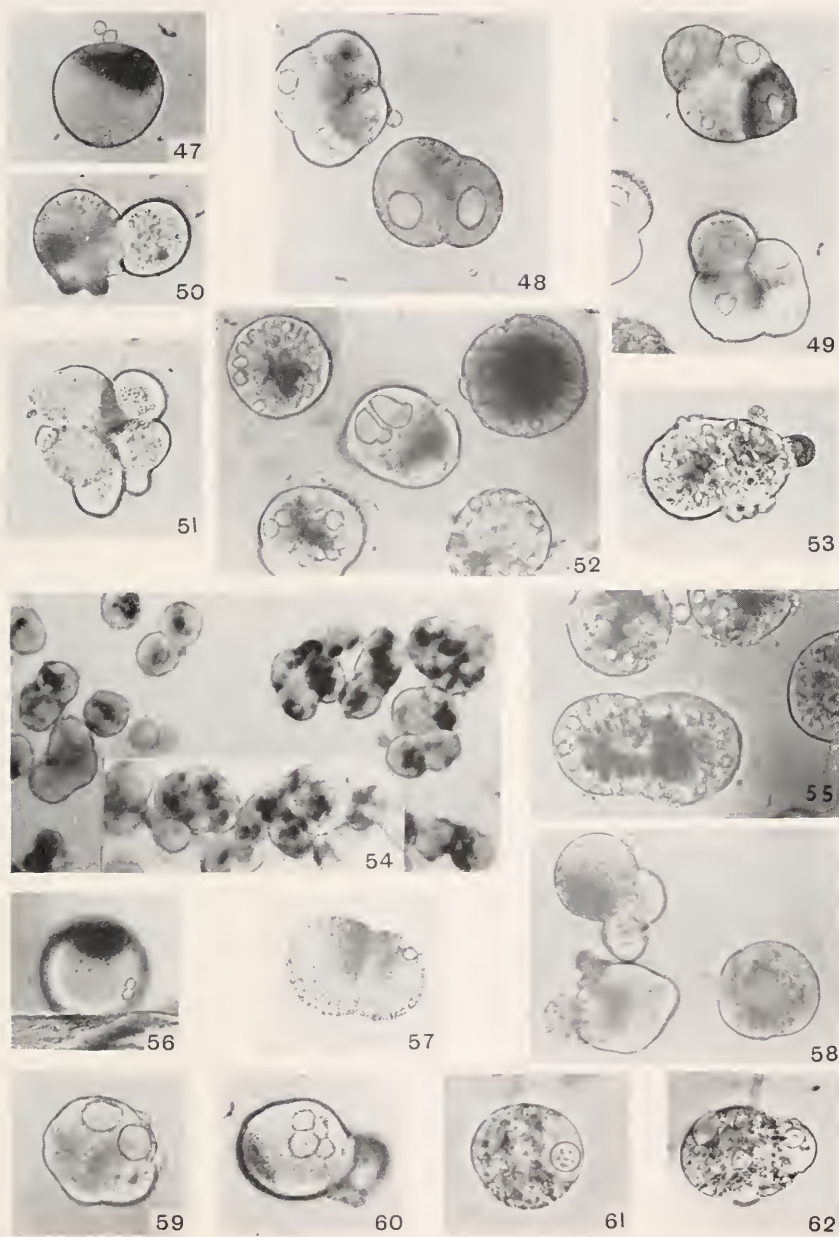
PHOTOGRAPH 57. Typical two-cell stage of parthenogenetic white half. Two hours after activation.

PHOTOGRAPH 58. Parthenogenetic white halves, amoeboid. One and one-half hours after activation. Cf. with Photograph 46 of whole centrifuged egg, parthenogenetic.

PHOTOGRAPHS 59, 60. Parthenogenetic white half blastulae, amoeboid, with irregular nuclei. Four hours after activation. Cf. with Photograph 52, fertilized white halves.

PHOTOGRAPHS 61, 62. Blastulae both ciliate and amoeboid. Twenty-four hours after activation. Cf. Photograph 62 with Photograph 53, fertilized white half.

PLATE IV



Photographs 47-62

were taken at short intervals; after two hours the egg ended up in a two-cell stage, much as it was in the beginning. The meaning of this is not known, but perhaps it points to a similar condition, with regard to the cell surface, for amoeboid motion and cell division.

Yellow Half-egg, Parthenogenetic (Parthenogenetic Merogone)

(Photographs 85-96)

The yellow half-eggs, containing no nucleus or polar spindle, may be activated artificially by KCl. These are the parthenogenetic merogones. After activation, a thick fluted membrane is lifted off exactly like that of the normal fertilized egg, and quite characteristic of this

PLATE V

Yellow half-eggs, fertilized. (Fertilized merogones)

PHOTOGRAPH 63. Sperm nucleus in yellow half-egg. Two hours after fertilization.

PHOTOGRAPH 64. Same egg with two nuclei, a few minutes later.

PHOTOGRAPH 65. Another yellow half-egg with several nuclei but no cell division. Five hours after fertilization.

PHOTOGRAPH 66. Two-cell stage; cells unequal.

PHOTOGRAPH 67. Typical two-cell stage. Two hours after fertilization.

PHOTOGRAPHS 68-75. Successive photographs of the same egg showing cleavage and fusion of blastomeres, and amoeboid character.

PHOTOGRAPH 68. Two equal cells. Two hours after fertilization.

PHOTOGRAPH 69. Five minutes later, 4 equal cells.

PHOTOGRAPH 70. Ten minutes later. Blastomeres begin to fuse.

PHOTOGRAPH 71. Three minutes later. Possibly polar lobe.

PHOTOGRAPH 72. One minute later. Three cells; two have fused.

PHOTOGRAPH 73. Six minutes later. Four (or 5) separate cells again.

PHOTOGRAPH 74. Five minutes later. Fusion of blastomeres, and egg becoming amoeboid. Compare with Photograph 45, of the whole centrifuged egg, parthenogenetic.

PHOTOGRAPH 75. One-half hour later, 3 hours after fertilization. Amoeboid. Compare with Photograph 46, of whole centrifuged egg, parthenogenetic; and with Photograph 58 of parthenogenetic white half and with Photograph 96 of parthenogenetic merogone.

PHOTOGRAPHS 76-84. Successive photographs at short intervals showing amoeboid character of a two-cell stage of the fertilized yellow half-egg.

PHOTOGRAPH 76. Two-cell stage, almost equal blastomeres. Fertilized at 9.30 A.M., photographed at 10.50 A.M.

PHOTOGRAPH 77, at 11.35 A.M.

PHOTOGRAPH 78, at 11.40 A.M.

PHOTOGRAPH 79, at 11.50 A.M.

PHOTOGRAPH 80, at 12 noon.

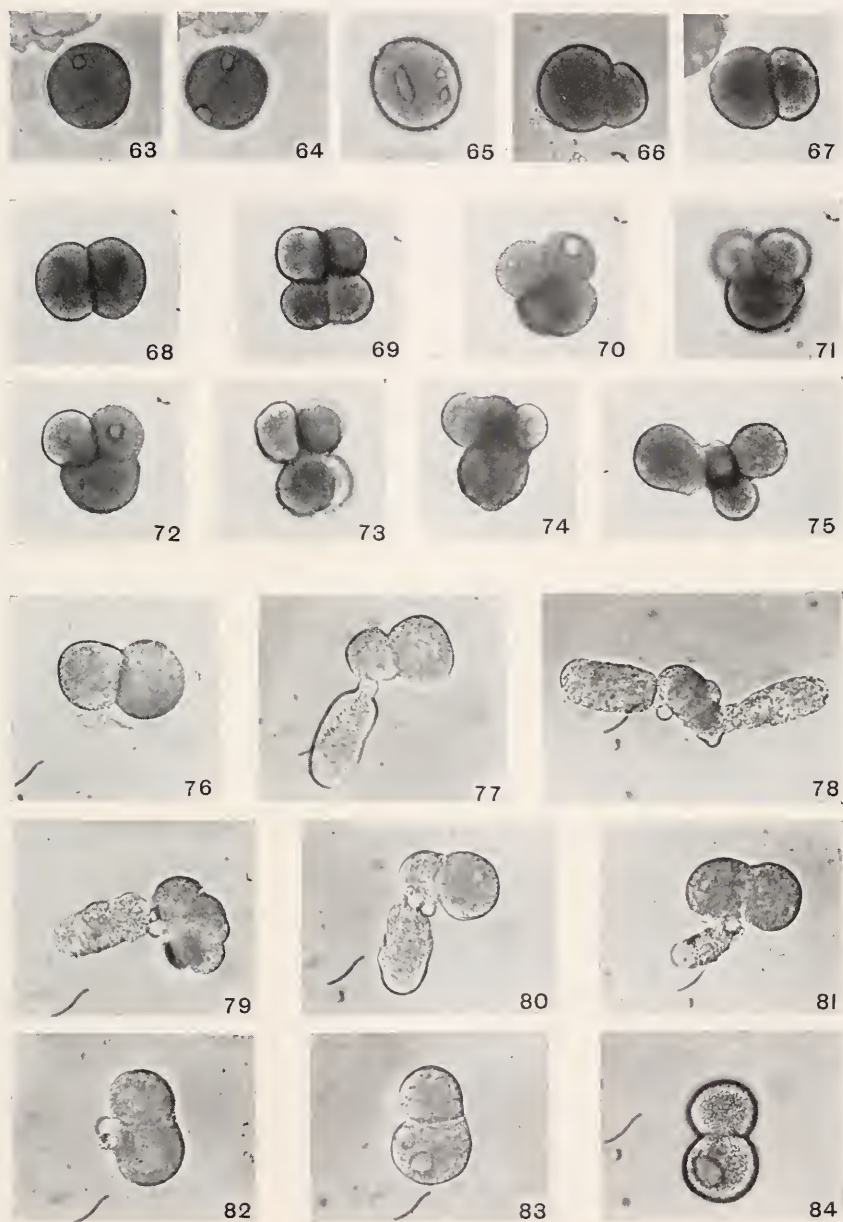
PHOTOGRAPH 81, at 12.10 P.M.

PHOTOGRAPH 82, at 12.20 P.M.

PHOTOGRAPH 83, at 12.21 P.M., two-cell stage, slightly unequal.

PHOTOGRAPH 84, at 1.10 P.M., two cells almost equal and quite similar to the original, Photograph 76. Note large nucleus.

PLATE V



Photographs 63-84

particular egg (Photograph 85, cf. Photographs 19, 21). They then usually become amoeboid and develop no further, though they may live for several days. There is no definite polar lobe, but amoeboid processes sometimes simulate it. Some cases of first cleavage have been observed, however, and these were of both the equal type and the unequal type with varying degrees of inequality (Photographs 86-90); these also became amoeboid and there was no further development (Photographs 91-93). The amoeboid activity of the parthenogenetic merogone is shown in a series of photographs taken at intervals of a few minutes (Photographs 94-96). The parthenogenetic merogone, then, resembles the fertilized merogone in its tendency to become amoeboid and in its limited cleavage, though the fertilized merogone goes perhaps one step further in development. (Compare Photographs 86-96 with Photographs 66-84).

Discussion

A study of the *Chactopterus* egg has shown that the non-nucleate fraction of the egg of another type of animal than the sea urchin can be activated artificially. The parthenogenetic merogone of *Chactopterus*

PLATE VI

Yellow half-eggs, parthenogenetic. (Parthenogenetic merogones)

PHOTOGRAPH 85. Yellow half, parthenogenetic, with characteristic fluted fertilization membrane. One hour after activation. At right upper corner is part of a whole cell, also with fluted membrane. Compare also with Photograph 19, of the normal whole egg.

PHOTOGRAPH 86. Two-cell stage, unequal. Two hours after activation. Compare with Photograph 66, of fertilized merogone.

PHOTOGRAPH 87. Typical two-cell stage. Two hours after activation. Compare with Photograph 67, of fertilized merogone.

PHOTOGRAPH 88. Cleaving into two almost equal cells. Five hours after activation.

PHOTOGRAPH 89. Two equal cells. Six hours after activation. Compare with Photograph 68 of fertilized merogone.

PHOTOGRAPH 90. Two equal cells. Five hours after activation. Compare with Photograph 76. Clear protrusion looks like a polar body.

PHOTOGRAPHS 91, 92, 93. Amoeboid forms of older parthenogenetic merogones. Twenty-four hours after activation. Compare with Photograph 46 of whole centrifuged egg, parthenogenetic, and with Photograph 75, of fertilized merogone.

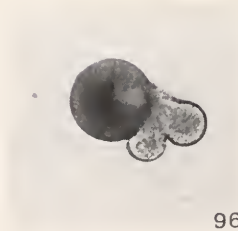
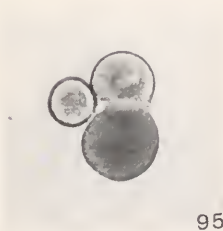
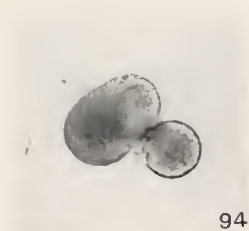
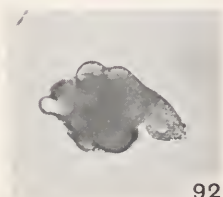
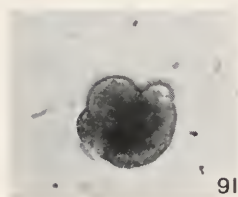
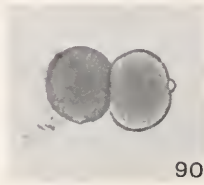
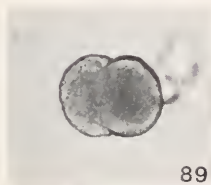
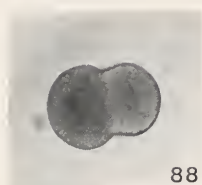
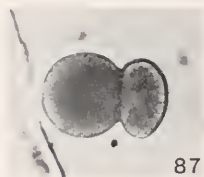
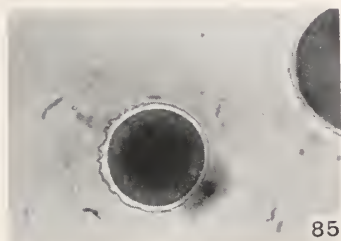
PHOTOGRAPHS 94-96. Successive photographs of the same parthenogenetic yellow half-egg, taken at very short intervals to show amoeboid character. Eighteen hours after activation. Compare with Photographs 76-84 of the fertilized merogone. Also compare Photograph 96 with Photograph 75 of the fertilized merogone, and with Photograph 46 of the parthenogenetic whole egg, and with Photograph 58 of the parthenogenetic white half.

PHOTOGRAPH 94. Activated at 5 P.M. July 21, photographed at 11 A.M., July 22.

PHOTOGRAPH 95. At 11.02 A.M.

PHOTOGRAPH 96. At 11.03 A.M.

PLATE VI



Photographs 85-96

goes through only one cleavage and thus does not develop as far as that of the sea urchin. This is not due, however, to the lack of a nucleus, since the same fraction fertilized does not develop much further. The unfertilized *Chactopterus* egg is perhaps more highly organized than the unfertilized sea urchin egg.

The parthenogenetic development of the normal *Chactopterus* egg is peculiar in that usually there is no orderly cleavage with the accompanying nuclear divisions, but instead, amoeboid forms with irregular cells and large nuclei. It is not surprising, therefore, that there is no orderly development of the parthenogenetic merogone, and that it likewise, becomes amoeboid. It may be that the concentration of heavy granules into this half of the egg is a factor also in preventing further development. This is suggested by the experiments of Whitaker and Morgan (1930), who obtained polar and anti-polar halves of the *Chactopterus* egg by cutting with a needle. These halves have, of course, a more even distribution of granules than the halves obtained by centrifuging. They found that after fertilization, both of the halves would cleave unequally like the whole egg, and that the anti-polar half would form a polar lobe; there were certain irregularities, however. But the anti-polar half obtained by cutting develops more normally, at least in the early stages, than the anti-polar half obtained by centrifuging. It is of interest, too, that the parthenogenetic merogone of the *Sphaerechinus granularis* egg, whose stratification is so similar to that of *Chactopterus* (Photographs 4 and 5) also becomes amoeboid and does not cleave regularly; the parthenogenetic centrifuged whole egg of this form likewise becomes amoeboid (see Photographs 53-56 of my 1938 paper).

The amoeboid activity which is characteristic of the parthenogenetic egg or its fraction both in *Chactopterus* and *Sphaerechinus*, may be largely due to the action of the parthenogenetic agent on the surface of the egg. Amoeboid activity may be another expression of the condition of the cell surface characteristic of cleavage just as gelation seems to be characteristic of some of the interior cytoplasm at this time, in the formation of asters.

That the early development is dependent on the cytoplasm rather than the nucleus is brought out again in the development of the white halves of the *Chactopterus* egg. These, whether fertilized or parthenogenetic, may cleave according to the peculiar and characteristic annelid pattern. It seems remarkable that these fractions, and even very much smaller fragments, according to Lillie and Wilson, may cleave just like the whole egg. This certainly leads one to the conclusion that it is the matrix or clear substance in the egg, rather than nuclei or visible granules that is important in early development.

Attention may be called to the frequent occurrence of equal cells in the first cleavage instead of the typical unequal cells. In the present experiments, there is a tendency to equal division in parthenogenetic whole eggs, both normal and centrifuged and both in the white and yellow halves both fertilized and parthenogenetic. Other observers have noted the same thing in certain of these cases. In the *Cumingia* egg, I found that pressure on the egg after fertilization had the same effect (Browne, 1910) and similar results have been obtained by Tyler (1930) for the *Chaetopterus* egg; he found also that cold, heat, ultra-violet rays and anaerobiosis would produce the same effect. One would assume that an unequal division of the two cells is a more specialized form, and that whatever mechanism is responsible for it, is put out of gear by certain experimental conditions. It seems to be due, at least in many cases, to an action especially on the surface of the cell, rather than on the interior.

Summary

1. The unfertilized *Chaetopterus* egg (94μ) may be stratified and broken into unequal halves of a uniform size by centrifugal force. The nucleate white halves (83μ) contain oil, clear layer, mitochondria and a little yolk. The non-nucleate yellow halves (64μ) contain only yolk.

2. The centrifuged whole egg may develop, both fertilized and parthenogenetic, similarly to the normal uncentrifuged egg. Certain peculiarities have been noted.

3. The white halves, both fertilized and parthenogenetic, may cleave according to the typical annelid pattern. But there is a tendency toward equal first cleavage and amoeboid form and later, development with irregular cells and large nuclei; the blastulae tend to fuse together; these peculiarities are much more pronounced in the parthenogenetic than in the fertilized white halves.

4. The yellow halves, both fertilized (= fertilized merogone) and parthenogenetic (= parthenogenetic merogone) usually lift off a characteristic fluted fertilization membrane and pass through only one cleavage and become markedly amoeboid.

5. Early development without nuclei (parthenogenetic merogony) is established for the annelid, *Chaetopterus*, in addition to the five species of echinoderms previously studied. The development of the parthenogenetic merogones of *Chaetopterus* does not go as far as that of the sea urchin.

LITERATURE CITED

- ALLYN, H. M., 1912. The initiation of development in Chaetopterus. *Biol. Bull.*, **24**: 21.
- BROWNE, E. N., 1910. Effects of pressure on Cumingia eggs. *Arch. f. Entw.-mech.*, **29**: 243.
- HARVEY, E. B., 1932. The development of half and quarter eggs of Arbacia punctulata and of strongly centrifuged whole eggs. *Biol. Bull.*, **62**: 155.
- HARVEY, E. B., 1936. Parthenogenetic merogony or cleavage without nuclei in Arbacia punctulata. *Ibid.*, **71**: 101.
- HARVEY, E. B., 1938. Parthenogenetic merogony or development without nuclei of the eggs of sea urchins from Naples. *Ibid.*, **75**: 170.
- LILLIE, F. R., 1902. Differentiation without cleavage in the egg of the annelid Chaetopterus pergamentaceus. *Arch. f. Entw.-mech.*, **14**: 477.
- LILLIE, F. R., 1906. Observations and experiments concerning the elementary phenomena of embryonic development in Chaetopterus. *Jour. Exper. Zool.*, **3**: 153.
- LILLIE, F. R., 1909. Polarity and bilaterality of the annelid egg. Experiments with centrifugal force. *Biol. Bull.*, **16**: 54.
- LOEB, J., 1901. Experiments on artificial parthenogenesis in annelids (Chaetopterus) and the nature of the process of fertilization. *Am. Jour. Physiol.*, **4**: 423.
- MEAD, A. D., 1895. Some observations on maturation and fecundation in Chaetopterus pergamentaceus, Cuvier. *Jour. Morph.*, **10**: 313.
- MEAD, A. D., 1897. The early development of marine annelids. *Ibid.*, **13**: 227.
- MEAD, A. D., 1898a. The origin and behavior of the centrosomes in the annelid egg. *Ibid.*, **14**: 181.
- MEAD, A. D., 1898b. The rate of cell division and the function of the centrosome. Biological Lectures delivered at the M. B. L. of Woods Hole, 1896-1897. Ginn & Co., Boston.
- MORGAN, T. H., 1937. The factors locating the first cleavage plane in the egg of Chaetopterus. *Cytologia*, Fujii Jubilee Volume, p. 711.
- MORGAN, T. H., 1938. A reconsideration of the evidence concerning a dorsoventral pre-organization of the egg of Chaetopterus. *Biol. Bull.*, **74**: 395.
- TYLER, A., 1930. Experimental production of double embryos in annelids and mollusks. *Jour. Exper. Zool.*, **57**: 347.
- WHITAKER, D. M., 1933. On the rate of oxygen consumption by fertilized and unfertilized eggs. IV. Chaetopterus and Arbacia punctulata. *Jour. Gen. Physiol.*, **16**: 475.
- WHITAKER, D. M. AND T. H. MORGAN, 1930. The cleavage of polar and anti-polar halves of the egg of Chaetopterus. *Biol. Bull.*, **58**: 145.
- WILSON, E. B., 1883. Observations of the early developmental stages of some polychaetous annelides. *Studies from the Biol. Lab., J. H. U.*, **2**: 271.
- WILSON, E. B., 1929. The development of egg-fragments in annelids. *Arch. f. Entw.-mech.*, **117**: 179.
- WILSON, E. B., 1930. Notes on the development of fragments of the fertilized Chaetopterus egg. *Biol. Bull.*, **59**: 71.

CHEMICAL MEDIATION IN CRUSTACEANS. III. ACETYLCHOLINE AND AUTOTOMY IN PETROLISTHES ARMATUS (GIBBES)

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Acetylcholine has been shown to be present in nervous tissues of crustaceans in considerable amounts (Welsh, 1938; Smith, 1939) and to have an excitatory action on the decapod heart (Welsh, 1939 *a* and *b*), but, thus far, its action elsewhere in crustaceans has not been demonstrated (see Katz, 1936, regarding acetylcholine and crustacean skeletal muscle).

Autotomy, or the casting of legs by crustaceans, is a well-known phenomenon resulting from a unisegmental, reflex stimulation of the autotomizer muscle of a leg (Wood and Wood, 1932). Hence central transmission at synapses, peripheral transmission at myoneural junctions, and conduction along fibers of the reflex pathway must occur in this process. If acetylcholine is normally involved in the transmission of nerve impulses in the autotomy reflex, it should be possible to obtain evidence by appropriate injections of acetylcholine and of drugs which are known to affect its rate of destruction and its action. That stimulating and inhibiting substances may play a part in autotomy in *Porcellana* and *Uca* was suggested by Hoadley (1934, 1937). Abramowitz and Abramowitz (1938), while testing a series of drugs for their effects on the chromatophores of *Uca*, observed that the injection of acetylcholine caused autotomy.

The present paper records observations made on autotomy in an anomuran of Bermuda. The experimental work was done at the Bermuda Biological Station and was aided by grants from the Milton and Porter Funds of Harvard University.

Materials and Methods

The animal used was *Petrolisthes armatus* (Gibbes), of the family Porcellanidae. The members of this family are said to autotomize with the greatest ease and after the least stimulus of any of the decapod crustaceans (Wood and Wood, 1932). *P. armatus* occurs in consid-

erable abundance under stones between tide levels in Bermuda. They were captured and handled with care in order to prevent autotomy, and experiments were performed, whenever possible, on animals which had been in the laboratory less than twenty-four hours.

Since *Petrolisthes* may be caused to autotomize a leg by grasping it gently with forceps, this was the method of stimulation employed. Individuals were placed in small containers filled with sea water. Each leg was seized, held for approximately one second, and, if not dropped, was then released. *Petrolisthes* has three pairs of legs which are developed for walking, and a pair of chelae, so a series of eight stimuli could be given the animal without repeated stimulation of those appendages which failed to autotomize on the first trial. Legs were grasped in random order and the order was varied from animal to animal.

After obtaining results on one hundred normal animals to serve as controls, fifty animals were injected at the base of one of the last walking legs with 0.05 cc. of a perfusion fluid prepared according to Pantin (1934). As the average body volume of the animals used was only 0.3 cc., this amount of injected fluid caused a considerable dilution of the blood, but this dilution and the act of injection had no evident effect on autotomy. The drugs employed were always given in the volume of fluid used in the injected controls. These drugs were acetylcholine chloride (Hoffman-LaRoche), eserine (physostigmine) sulphate (Merck), atropine sulphate (Merck), and adrenalin chloride (Parke-Davis). Fresh solutions of these substances were prepared daily and the necessary dilutions were made just before using. This is especially important in the case of acetylcholine which is rapidly hydrolyzed in an alkaline solution.

Results

Control Experiments.—The results obtained on uninjected control animals are given in Fig. 1. The first leg¹ stimulated by grasping was dropped by 54 of the 100 animals, or it may be said that there was 54 per cent autotomy. The second, third and fourth legs stimulated showed increasing tendency to autotomize up to 85 per cent. Then an abrupt drop in the number of autotomies appeared until the eighth, or last leg, autotomized in only 21 per cent of the trials. This increasing tendency to autotomize up to a certain point, followed by a decreasing tendency, is not due to individual differences in the autotomizing mechanisms of different legs, for with such differences alone a varying, random order of stimulation would give a straight line parallel to the abscissa. It

¹ No distinction will be made between walking legs and chelae and both types of appendages will be spoken of as legs.

can be explained, however, by assuming that each stimulus to a leg, whether the leg is dropped or not, affects the tendency of the other legs to autotomize. Up to the fourth stimulus the effect is excitatory and after the fourth stimulus it is inhibitory.

Fifty *Petrolisthes* injected with 0.05 cc. of "crab Ringer" and stimulated ten to fifteen minutes after injection autotomized in a manner similar to the uninjected controls (Fig. 1).

The line which is drawn through the data shown in Fig. 1 may be called the normal curve of autotomy for *Petrolisthes*, when stimulation

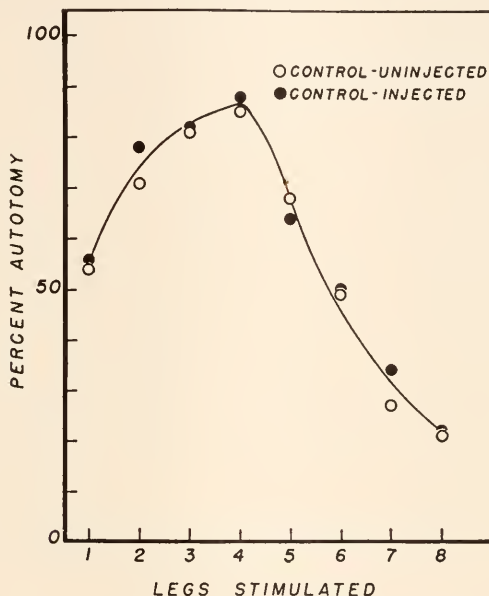


FIG. 1. Percentage of legs dropped by 100 normal animals when each leg was stimulated once, and the order of stimulation was varied from one animal to the next, is shown by the open circles. Results obtained on 50 animals injected with 0.05 cc. of "crab Ringer" are shown by the closed circles. The line drawn through the data has been called the "normal curve" of autotomy.

is as described. An obvious change in the "normal curve" produced by the injection of some substance would give an indication of the effect of that substance on the process of autotomy.

Acetylcholine.—The injection of various amounts of acetylcholine in preliminary tests indicated that 25 gamma of acetylcholine² was intermediate between a lethal dose and one whose effects had largely disappeared between the time of injection and the time of stimulation. In

² The amount of substance injected will be expressed as the weight of the salt even though the salt is not indicated. The volume of fluid was always 0.05 cc.

most cases larger amounts caused a number of legs to be dropped during the injection process, or shortly after the injection was made. The legs dropped were almost always on the side on which the injections were made.³ In 20 per cent of the animals the injection of 25 gamma of acetylcholine caused one to three legs to be dropped without further stimulation.

Fifty animals, each of which was injected with 25 gamma of acetylcholine and its legs stimulated approximately five minutes after injection, yielded results which are shown in Fig. 2. The first leg to be grasped

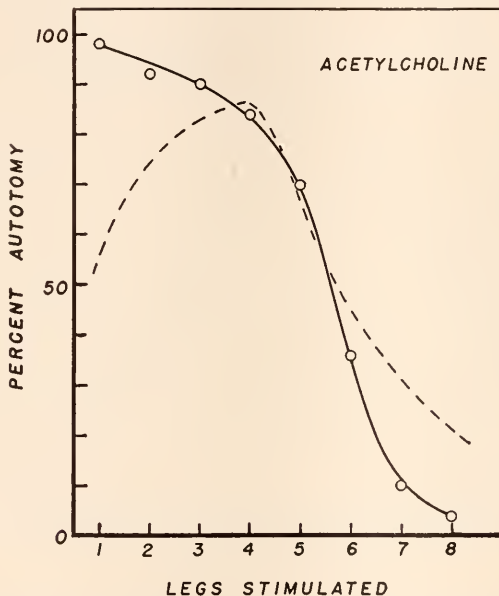


FIG. 2. Percentage autotomy in 50 animals each of which received 25 gamma of acetylcholine chloride five minutes before stimulation of the legs. The dashed line in this and the following figures is the "normal curve" of autotomy.

was dropped in 98 per cent of all the animals, but immediately an "inhibitory" phase appeared and the entire curve resembles the inhibitory part of the "normal curve." It is apparent that the injection of acetylcholine markedly affects the autotomizing process and, at this concentration, eliminates the period of increasing tendency to autotomize.

Eserine.—*Petrolisthes* was found to be remarkably sensitive to eserine. The injection of 0.5 gamma or more caused, after a delay of one

³ The injection of 100 gamma of acetylcholine into each of six *Pachygrapsus transversus* (Gibbes), a rock crab, caused the autotomy of three to five legs, on the side of the injection, during the injection process. In two cases all of the legs on the opposite side were autotomized within a few seconds after injection.

- to two minutes, extreme activity followed by tetanic convulsions and death. The injection of 0.25 gamma caused greatly increased general activity and uncoordinated movements followed by sluggishness, failure to autotomize, and the death of some animals. A dose of 0.1 gamma caused increased activity for a few minutes after injection, but following this the reactions of the majority of the animals appeared normal. This amount was administered to fifty animals in groups of ten, and ten to fifteen minutes later their legs were stimulated as were those of the controls. The results may be seen in Fig. 3. The eserine curve is quite

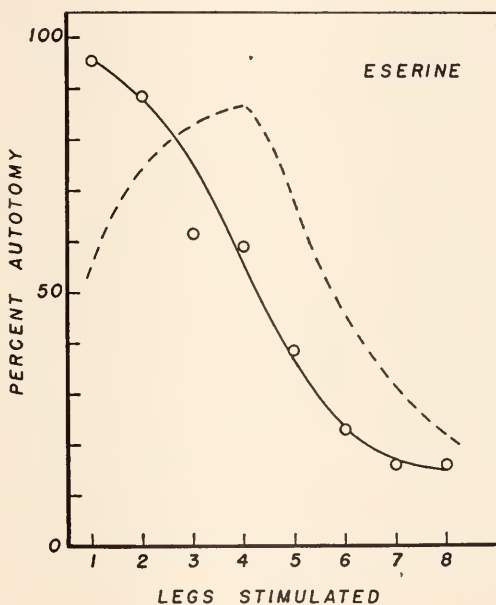


FIG. 3. Percentage autotomy in 50 animals injected with 0.1 gamma of eserine sulphate and their legs stimulated ten to fifteen minutes after injection.

similar to the one obtained for acetylcholine, except that it is slightly lower and shifted somewhat to the left due to a lower percentage of autotomies in all except the last two legs to be stimulated. The important points, however, are the absence of a period of increasing tendency to autotomize, and the large number of autotomies (95 per cent) of first legs to be stimulated.

Atropine.—Relatively large doses of atropine had to be injected before definite effects were obtained. Amounts above 100 gamma caused marked sluggishness and prevented all autotomies during a period of fifteen minutes after injection. A dose of 100 gamma resulted in de-

creased general activity for a period of five minutes after the injection but ten minutes later the animals appeared normal, hence this was selected as the dose to be employed. Eighty animals were injected in lots of ten, and ten minutes later their legs were stimulated. Fifteen animals failed to drop any legs and in the calculations of percentage of autotomies these were omitted. The remaining sixty-five animals showed a marked reduction in the total number of legs dropped, as may be seen in Fig. 4. Only 46 per cent dropped a leg at the first stimulus and this was followed by a decline in the number of autotomies with

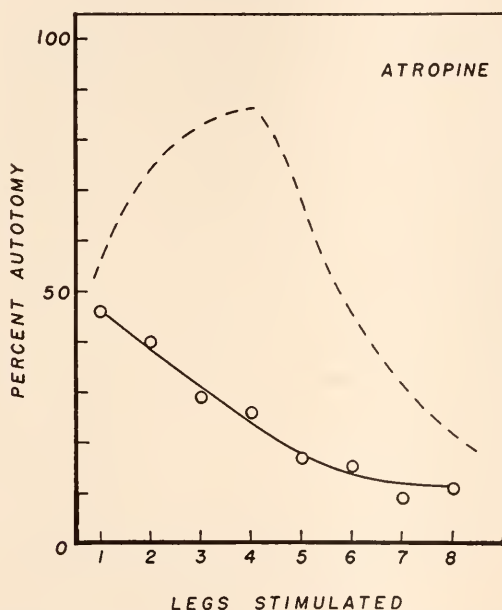


FIG. 4. Percentage autotomy in 65 animals injected with 100 gamma of atropine sulphate and their legs stimulated ten minutes after injection.

subsequent stimuli. Thus atropine inhibits autotomy more or less completely, depending on the amount injected.

Adrenalin.—Adrenalin (adrenine) is known to have an excitatory action on the decapod heart (Bain, 1929; Welsh, 1939a and b), therefore it seemed desirable to test its effects on autotomy in *Petrolisthes*. Adrenalin chloride was injected in amounts varying from 1 gamma to 50 gamma per animal. A dose of 50 gamma produced marked inactivity, uncoordinated movements and occasionally death. A dose of 1 gamma had no noticeable effects, either on general behavior, or on autotomy. Fifty animals injected with 2.5 gamma of adrenaline and stimulated

ten to fifteen minutes later gave results which are shown in Fig. 5. The shape of the curve is not very different from the "normal curve," but its displacement indicates that adrenalin, in this concentration, has a slight inhibitory effect over the entire series of legs stimulated.

Autotomy in Males and Females.—The following observations have only an indirect bearing on the general question of chemical mediation and autotomy, but seem worthy of mention. Evidence presented by Hoadley (1934; 1937) indicated that in *Uca* and *Porcellana*, females without eggs and males tended to autotomize more readily than did fe-

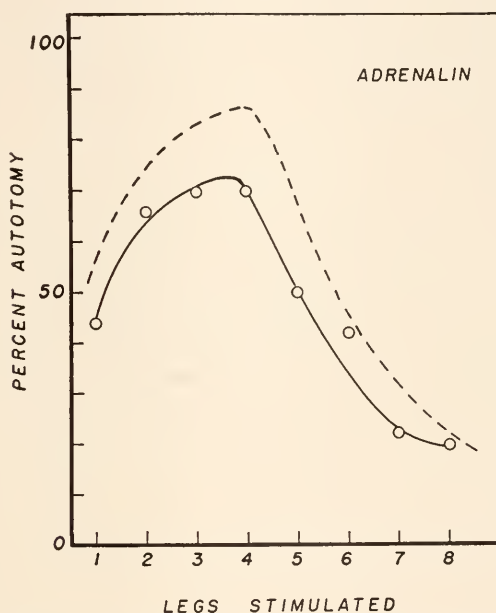


FIG. 5. Percentage autotomy in 50 animals injected with 2.5 gamma of adrenalin chloride and their legs stimulated ten to fifteen minutes after injection.

males with eggs. Our data on control animals, including one group of one hundred animals which have not been mentioned previously, were examined to see if *Petrolisthes* showed similar differences. The great majority of the females were carrying eggs at the time our observations were made; hence the results for the small group not carrying eggs are of little significance. Table I includes the data from 249 animals. It is apparent from these data that the tendency to autotomize in *Petrolisthes* is not modified by the carrying of eggs. However, this does not preclude the possibility of there being such a modification in other groups of crustaceans.

Discussion

It has been shown (1) that stimulation of the legs of *Petrolisthes armatus* produces changes in the animal which result in an increasing percentage of autotomies for the first four legs followed by a progressive decrease in the number of autotomies for the remaining legs; (2) that the injection of acetylcholine facilitates or causes autotomy; (3) that eserine, which prevents the enzymic hydrolysis of acetylcholine, produces certain effects similar to those produced by injected acetylcholine; (4) that atropine, which prevents the muscarin-like action of acetylcholine, causes a partial or complete reduction in the number of autotomies. This evidence indicates that acetylcholine normally acts in the autotomy reflex of *Petrolisthes*. Interesting questions are: (1) where and in what way does this action occur, and (2) why does the stimula-

TABLE I

	Males		Females with eggs		Females without eggs	
	No. of animals	Average no. legs dropped per animal	No. of animals	Average no. legs dropped per animal	No. of animals	Average no. legs dropped per animal
Regular order of stimulation	52	4.50	45	4.38	3	5.0
Random order of stimulation	49	4.44	48	4.95	3	5.0
Injected controls	23	4.74	22	5.13	4	5.0

tion and autotomy of a leg affect the tendency of remaining legs to be dropped? These and other questions cannot be definitely answered with the evidence at hand but certain suggestions, to be tested by further experiments, may be offered.

Although the ventral ganglia of *Petrolisthes* are fused into a single mass, as in the Brachyura, there is an anatomical arrangement according to a segmental pattern. Stimulation of a leg by pinching excites sensory fibers to the ventral ganglion supplying that segment. Internuncial neurones carry the impulses to the motor fibers which innervate the autotomizer muscle of that leg. Since it is extremely rare for any leg to be dropped other than the one being stimulated, it is apparent that any impulses going out from a ganglion to other ganglia must ordinarily be below threshold for the autotomizer muscles of other legs. However, sub-threshold impulses might produce facilitation, as has been shown by

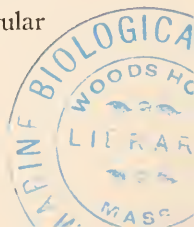
Pantin (1934; 1936*a* and *b*) for leg muscles of *Carcinus*, and the facilitating process might be the release of acetylcholine at motor nerve endings. There is good evidence that acetylcholine is present in crustacean blood in considerable amounts (Smith, 1939). Its accumulation, as a result of successive stimuli, might account for the regular increase in the number of autotomies of the first four legs stimulated in the control animals. Why, with subsequent stimuli, there should then be a regular decrease in the number of autotomies of the remaining legs is not readily explainable. In accounting for certain phenomena associated with Wedensky inhibition and fatigue in vertebrate muscle, Rosenblueth and Morison (1937) have postulated that high concentrations of acetylcholine are paralytic. Possibly after the fourth leg of *Petrolisthes* is stimulated the acetylcholine concentration in the blood reaches a paralytic range and inhibition then sets in. However, if this were the case, on the basis of the control data one would expect that 85 per cent autotomies would be the maximum obtainable before the paralytic threshold of acetylcholine were reached; instead, injections of acetylcholine and eserine may result in as high as 98 per cent autotomies.

Rosenblueth and Morison (1937) have suggested that in high frequency stimulation of vertebrate nerve the quantal yield of acetylcholine, per impulse, is greatly reduced as stimulation is continued. A similar situation may occur in *Petrolisthes* as successive volleys of impulses traverse the reflex pathways. Taking into consideration the rate of production and the rate of destruction of the acetylcholine it follows that its concentration in the general circulation would rise to a maximum followed by a sharp decline as stimulation is continued. If, then, the degree of facilitation of autotomy is directly dependent on the concentration of acetylcholine in the region of the autotomizer muscles, one would expect precisely the type of curves obtained from the control animals and from those injected with acetylcholine. Additional data must be obtained, however, before this explanation is entirely acceptable.

What appears to be a clearly demonstrable rôle of acetylcholine in autotomy in crustaceans is of interest in itself but of greater interest is the evidence that acetylcholine may, after all, be the transmitter or mediator substance between motor nerves and skeletal muscle of crustaceans.

Summary

1. A single stimulus to each of the walking legs and chelae of the anomuran, *Petrolisthes armatus*, was found to cause, in most animals, the autotomy of four or more of these eight appendages. The appendages least often dropped were the first, sixth, seventh, and eighth of a series, when the order of stimulation was a random one. A regular



increasing tendency to autotomize in the first four legs was followed by a decreasing tendency in the remainder.

2. Injection of acetylcholine in relatively high concentrations caused the autotomy of one to several legs without further stimulation. A concentration was found which only occasionally caused autotomy when injected, but which facilitated the autotomy of the first three legs to be stimulated and eliminated, thereby, the period of increasing tendency to autotomize.

3. The injection of eserine caused a marked increase in general activity and a relatively small amount (0.1 gamma per animal) facilitated the autotomy of the first and second legs. The injection of eserine never caused legs to be dropped unless they were stimulated, otherwise its effects on autotomy were very similar to those produced by acetylcholine.

4. Atropine caused a general lowering of excitability and in relatively high concentrations completely prevented autotomy.

5. The injection of adrenalin was followed by a reduction in the percentage of autotomies of each of the eight legs but the phases of increasing and decreasing "excitability" remained unchanged.

6. No differences, correlated with sex or the bearing of eggs, were found in the average number of autotomies per individual.

7. The evidence obtained has been interpreted as indicating that acetylcholine normally plays a rôle in the autotomy reflex of *Petrolisthes* probably by acting as a mediator of impulses between the motor nerve and autotomizer muscle of a leg.

LITERATURE CITED

- ABRAMOWITZ, A. A., AND R. K. ABRAMOWITZ, 1938. On the specificity and related properties of the crustacean chromatophorotropic hormone. *Biol. Bull.*, **74**: 278.
- BAIN, W. A., 1929. The action of adrenaline and of certain drugs upon the isolated crustacean heart. *Quart. Jour. Exper. Physiol.*, **19**: 297.
- HOADLEY, L., 1934. Autotomy in the anomuran, *Porcellana platycheles* (Pennant). *Biol. Bull.*, **67**: 494.
- HOADLEY, L., 1937. Autotomy in the brachyuran, *Uca pugnax*. *Biol. Bull.*, **73**: 155.
- KATZ, B., 1936. Neuro-muscular transmission in crabs. *Jour. Physiol.*, **87**: 199.
- PANTIN, C. F. A., 1934. On the excitation of crustacean muscle. I. *Jour. Exper. Biol.*, **11**: 11.
- PANTIN, C. F. A., 1936a. On the excitation of crustacean muscle. II. Neuro-muscular facilitation. *Jour. Exper. Biol.*, **13**: 111.
- PANTIN, C. F. A., 1936b. On the excitation of crustacean muscle. IV. Inhibition. *Jour. Exper. Biol.*, **13**: 159.
- ROSENBLUETH, A., AND R. S. MORISON, 1937. Curarization, fatigue and Wedensky inhibition. *Am. Jour. Physiol.*, **119**: 236.
- SMITH, R. I., 1939. Acetylcholine in the nervous tissues and blood of crayfish. *Jour. Cell. Compar. Physiol.* In press.

- WELSH, J. H., 1938. Occurrence of acetylcholine in nervous tissues of crustaceans and its effect on the crab heart. *Nature*, **142**: 151.
- WELSH, J. H., 1939a. Chemical mediation in crustaceans. I. The occurrence of acetylcholine in nervous tissues and its action on the decapod heart. *Jour. Exper. Biol.*, **16**: 198.
- WELSH, J. H., 1939b. Chemical mediation in crustaceans. II. The action of acetylcholine and adrenalin on the isolated heart of *Panulirus argus*. *Physiol. Zool.* In press.
- WOOD, F. D., AND H. E. WOOD, II, 1932. Autotomy in decapod Crustacea. *Jour. Exper. Zool.*, **62**: 1.

SEXUAL PHASES IN TERRESTRIAL NEMERTEANS

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There has been much confusion in the literature regarding the sexuality of the various species of terrestrial nemerteans, due partly to the small number of individuals that have been available for study and partly to the fact that in some cases only the older and larger individuals have been collected. Since some of the species have a distinct tendency toward protandry and some are irregularly hermaphroditic, the successive stages in the life history are necessary in order to determine the potential sexuality of the individual.

Particularly confusing have been the accounts of the sexual conditions in *Geonemertes palaensis*, a species found on Pelew (Palao), Samoa and several other tropical Pacific islands. A collection consisting of 21 representatives of this species recently submitted to the writer for study included individuals representing such a series of sexual phases as will give a presumably reliable clue to their sequence. This occasion has also been taken to re-examine the consecutive sexual phases of the Bermuda land nemertean (*G. agricola*) as described many years ago (Coe, 1904).

The 12 apparently valid species of terrestrial nemerteans which have been described up to the present time all belong to the genus *Geonemertes*. Three of these, *G. australiensis* Dendy, *G. hillii* Hett, and *G. rodericana* (Gull.) are described as of separate sexes and oviparous, with the males usually smaller than the females. Three others, *G. dendyi* Dakin, *G. graffi* Bürger and *G. novae-zelandiae* Dendy have been thought to be of separate sexes, although no males have yet been found. One species, *G. spirospemia* Darbshire, is known only from a single male and another, *G. caeca* Darbshire, from a single female. *G. chalicophora* Graff was first described as hermaphroditic, although Böhmig (1898) found only females. *G. palaensis* Semper is hermaphroditic, as originally described and as found by von Kennel (1878), but only ovaries were present in the single specimen studied by Schröder (1918) as well as in the five investigated by Hett (1928). *G. arboricola* Pun-

¹ Contributions from the Scripps Institution of Oceanography. New Series, No. 55.

nett is likewise hermaphroditic and, as pointed out by Hett (1928), resembles *G. palaensis* so closely as to suggest specific identity.

All the above are known or assumed to be oviparous but the land nemertean of Bermuda, *G. agricola* (Will. Suhm), has been shown to be irregularly protandric, hermaphroditic and viviparous (Coe, 1904). The egg clusters of *G. australiensis* have been described by Dendy (1893) and those of *G. dendyi* by Waterston and Quick (1937). Also one of the specimens of *G. palaensis* studied by Hett (1928) had an egg cluster attached to the proboscis.

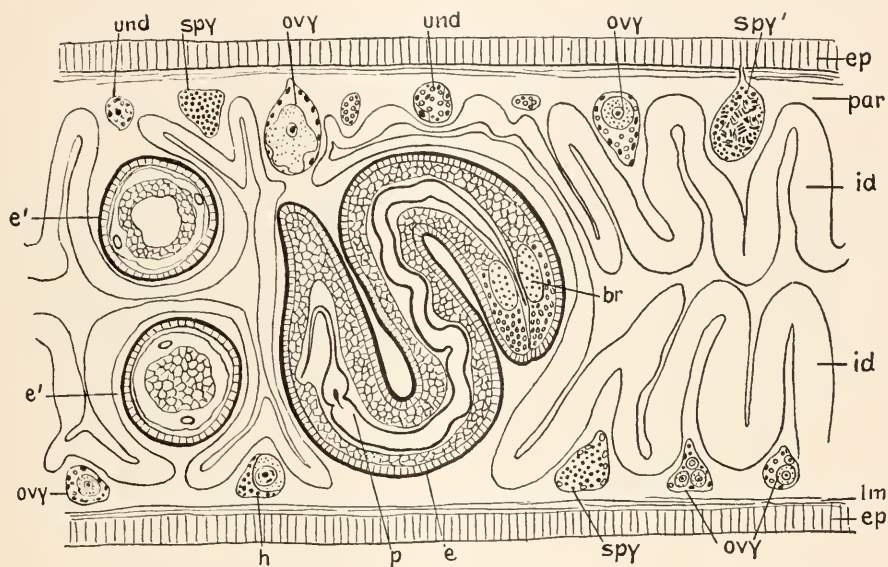


FIG. 1. *Geonemertes agricola*. Portion of horizontal section of body dorsal to nerve cords, showing sections of two embryos (*e*, *e'*) nearly ready for birth. Small gonads representing young ovaries (*ovy*), young spermaries (*spy*), spermaries with ripe spermatozoa (*spy'*), gonads of the hermaphroditic type (*h*), as well as others still remaining undifferentiated (*und*) are already present in anticipation of the following sexual phases. Other letters represent: *br*, brain of embryo; *ep*, epithelium of body wall; *id*, intestinal diverticula; *lm*, longitudinal musculature of body wall; *p*, proboscis of embryo, *par*, parenchyma. Slightly diagrammatic.

SEXUAL PHASES IN *G. AGRICOLA*

The study of more than a hundred individuals representing all ages of *G. agricola* shows that the young worms are usually protandric, since at first sexual maturity they have their bodies distended with spermaries. After the discharge of the sperm or sometimes earlier, ovaries begin to develop and the ovocytes slowly grow to maturity in the first female

phase. Before the eggs are ready for fertilization, however, other gonads in the same individual may develop rapidly into spermaries, and some of these may form functional spermatozoa in time to fertilize the eggs of the same individual or of another individual which may be in a similar female phase.

Shortly after fertilization the oviducts are closed and the eggs undergo development. In some individuals only a few embryos are formed (Fig. 1) but in others the numbers are so great that the mother's body

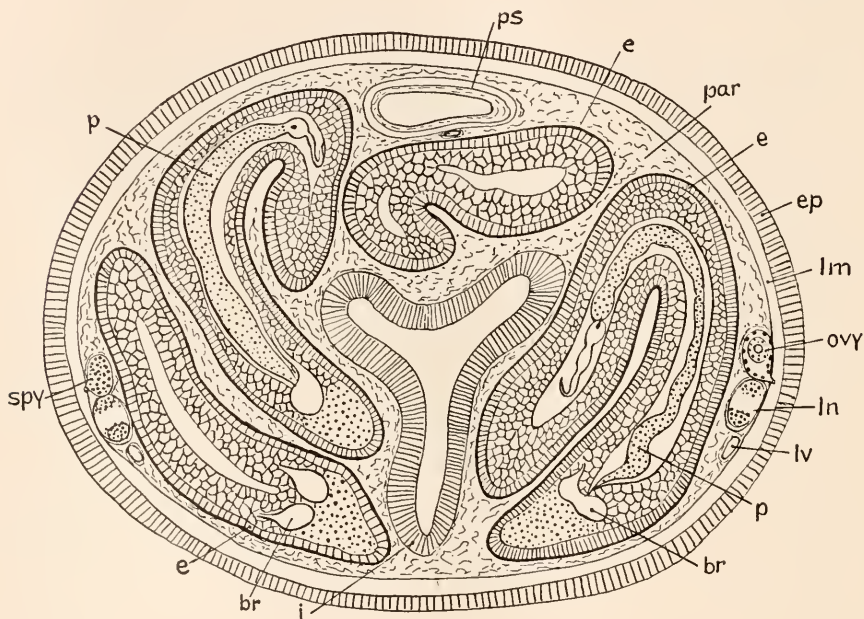


FIG. 2. *Geonemertes agricola*. Transverse section of body distended with embryos of which four have been cut in this section; showing also small ovary (*ovy*) and small spermary (*spy*); *i*, intestine; *ps*, proboscis sheath; other letters as in Figure 1. Slightly diagrammatic.

becomes enormously distended (Fig. 2). After all the organ systems of the adult except the reproductive organs have been acquired the young worm forces its way out of the mother's body by rupturing her body walls.

Even while embryos remain in the body, preparations are in progress for the succeeding sexual phases, both spermaries and ovaries continuing to be differentiated (Fig. 1).

After the discharge of the embryos such spermaries as have not previously been active now become functional as the second male phase.

This is later followed by the second female phase, with some new spermaries becoming functional when the ova are fully ripe.

The primary male phase is thus followed by a female phase which often changes to a functional hermaphroditic phase before the ova are ready for fertilization. Then follows a series of male and female (or hermaphroditic) phases, probably with seasonal interruptions of considerable duration, throughout the lifetime of the individual. There is much individual variation, however, in regard to the relative preponderance of male and female characteristics and time of maturity of each type of sexual products, since some individuals in the female or hermaphroditic phase have many large ovaries and few spermaries; others have

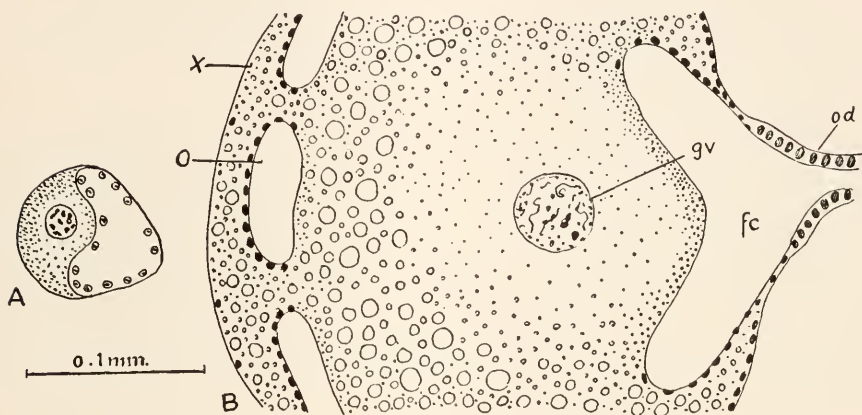


FIG. 3. *Geonemertes agricola*. A, young ovary with single ovocyte and associated follicle cells lining ovarian cavity. B, nearly mature ovary, showing portion of the single ovum with part of original ovarian cavity converted into a fertilization chamber (fc) continuous with the oviduct (od); x, peripheral cytoplasm; o, extensions of ovarian cavity lined peripherally with yolk-forming follicle cells; gv, germinal vesicle surrounded by yolk-free cytoplasm. Scale indicates magnification.

a true female phase with no spermaries at the time. Variations in the proportion of primary gonads which are to become differentiated into the two types of sexual glands and differences in the time of their development would account for the diversity of sexual conditions observed.

Differentiation of Gonads

It has been previously reported (Coe, 1904) that the gonads, all of which are primarily ambisexual in nature, become differentiated into three types before maturity. In those which are to form ovaries the ovocyte increases rapidly in size, first by assimilating smaller potential ovocytes and spermatogenic cells (if present) and later by yolk-forma-

tion through the activities of the follicle cells in the peripheral layer of cytoplasm (Fig. 3) as described more fully in the account of oogenesis in *G. palaensis* in the following section of this report. In the spermaries the potential ovocytes are inhibited in their growth by the multiplication of spermatogonia.

Relatively few of the gonads retain their primary ambisexual character to maturity by the harmonious development of both types of gametes. These few hermaphroditic gonads apparently discharge their spermatozoa previous to yolk-formation by the ovocyte. In other cases the ovocyte in such gonads undergoes fragmentation and cytolysis following the discharge of the sperm.

The ripe ova are from 0.35–0.45 mm. in diameter, or from one-fourth to one-half as great as the diameter of the entire body of the worm. Development is of the direct type (Coe, 1904).

SEXUAL PHASES IN *G. PALAENSIS*

This species appears to be normally oviparous as evidenced by the finding of an egg cluster by Hett (1928). The material now available for study shows that the sexual phases are similar to those reported previously for *G. agricola* (Coe, 1904) and summarized above. The primary functional male phase is followed similarly by successive female (potentially hermaphroditic) and male phases, the extent of hermaphroditism depending on the time of development of the spermaries and their relative number.

Of the 21 specimens in this collection 2 were in the functional male phase at the time of collection, with ripe spermaries and immature ovaries; 3 were functionally female at the time of collection, with ovaries showing various stages of oogenesis but no spermaries; 8 were unripe sexually with small ovaries and undeveloped spermaries; and 8 were functional hermaphrodites with large ova and ripe or nearly ripe spermaries.

The potentially hermaphroditic phases may in certain individuals or at certain periods in the sexual sequence be predominantly male or predominantly female or exclusively functional as one sex or the other. A greater number of individuals will, however, be found in the female phase than in the male phase of sexuality, since the time required for the development of the ovaries is much greater than for the spermaries. Consequently, if the collectors had obtained only such of these specimens as were in the exclusively female phase at the time of collection, it would have been concluded that the species must be of separate sexes. It is suspected that this may be the case with some of the other species which

are known from only one or a few specimens and in which neither protandry nor hermaphroditism has been observed.

The males or the primary male-phase individuals, whichever they may be, are smaller than the functional females and consequently less

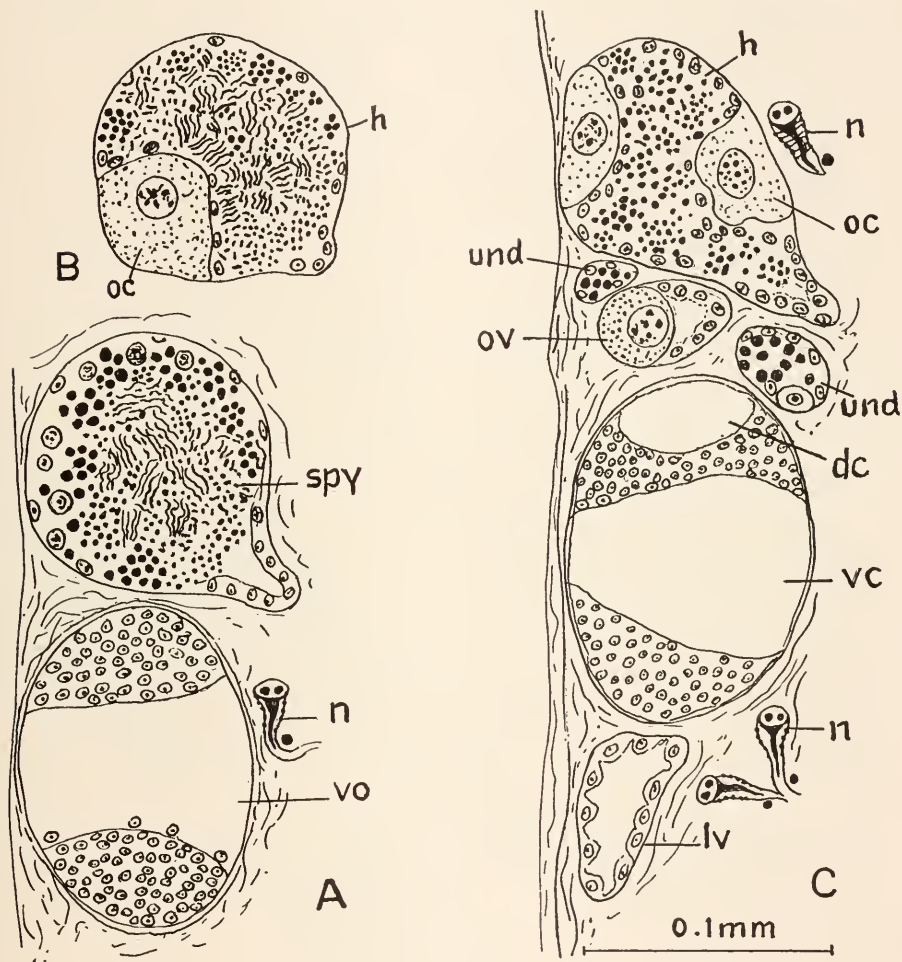


FIG. 4. *Geonemertes agricola*. A, transverse section of lateral nerve cord (vo) and adjacent spermary (spy) from individual containing fully grown embryos; n, a single flame cell of nephridial system. B, hermaphroditic gonad with single ovocyte (oc) from same individual. C, *Geonemertes palacensis*. Portion of transverse section of body, showing hermaphroditic gonad (h) with spermatids and two small ovocytes (oc), one small ovary (ov) with ovocyte and follicle cells, also two undifferentiated gonads (und). Other letters as in Figure 5. Scale beneath drawing shows magnification.

likely to be found by collectors. In *G. dendyi*, for example, no male or male-phase individual has yet been reported although females have been found by Dakin (1915) in the natural habit of the species, in Aus-

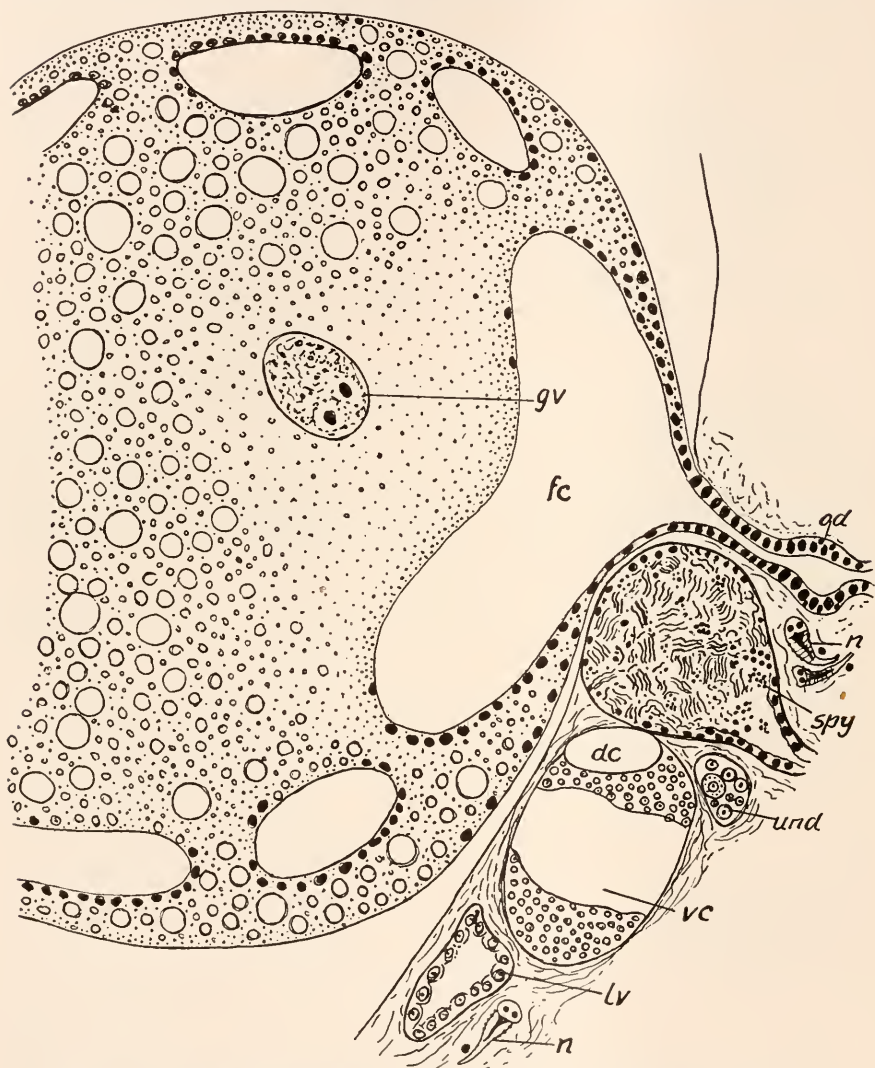


FIG. 5. *Geonemertes palaensis*. Portion of transverse section of body, showing fully ripe spermary (*spy*) and mature ovum ready for fertilization, also a small, undifferentiated gonad (*und*). Other letters indicate: *fc*, portion of original ovarian cavity, now converted into the fertilization chamber continuous with the oviduct (*od*); *gv*, germinal vesicle; *dc* and *vc*, dorsal and ventral cores of lateral nerve cord; *lv*, lateral blood vessel; *n*, flame cells of nephridial system. Same magnification as Fig. 4.

tralia, and by Stammer (1934) in Germany and Waterston and Quick (1937) in Wales, into which countries it has been introduced. Stammer has suggested the possibility that all the terrestrial species are protandric hermaphrodites. Such proves to be the condition in the two species which form the basis of the present paper but the extent to which the suggestion may apply to other species can be determined only after their life histories have been more completely investigated.

Ovogenesis

The young ovary usually has a single ovocyte on the wall nearest the center of the body, with a number of small follicle cells lining the ovarian cavity (Figs. 4, 5). As the ovocyte increases in size it becomes partially separated from the ovarian wall by wide spaces continuous with the ovarian cavity, eventually remaining attached to the wall by broad pseudopodia only. These pseudopodia then spread out and flow together distally to form a continuous cytoplasmic lining on the ovarian wall. This peripheral cytoplasm becomes widely separated from the main body of the ovocyte by these extensions of the ovarian cavity but remains united with it by large protoplasmic connections (Fig. 5). The surface of the ovum is thereby greatly increased.

Meanwhile the follicle cells have multiplied rapidly and spread as a continuous layer of tissue covering the peripheral cytoplasm and now function in the formation of yolk. As rapidly as the yolk is formed it is carried by cytoplasmic streaming into the main portion of the rapidly growing ovocyte. With the completion of yolk-formation, the peripheral cytoplasm is drawn into the ovocyte which then assumes a nearly spherical form.

Before the egg is mature the follicle cells lining the walls of the distal ovarian chamber form a continuous, epithelial-like layer which grows distally through the connective tissue and muscular layers of the body wall to form the oviduct (Figs. 4, 5). When the ovum is fully ripe the oviduct is completed through the surface epithelium of the body and provides an opening through which sperm may enter and through which, in the oviparous species, the egg will be discharged later. The remaining ovarian cavity provides a fertilization chamber through which the sperm passes before entering the yolk-free animal pole of the ovum (Fig. 5). The presence of two mature ova in a single ovary is very unusual in *G. palaensis* but occurs occasionally in *G. agricola*. In the young ovaries, however, two or more ova are of common occurrence (Fig. 4) but all except one of these are ingested or cytolysed during ovogenesis. The yolk, however, is formed more largely by the activities of the follicle cells than by the ingestion of the smaller ovocytes.

GENERAL DISCUSSION

Development of Gonads

In the hermaphroditic species three types of gonads are often present in the same individual: (a) spermaries, (b) hermaphroditic gonads which have retained their early ambisexual character and contain both sperm-forming cells and immature ovocytes, and (c) ovaries. The relative proportion of each of these three types at each sexual phase is highly variable. Except in young individuals the ovaries are much more numerous than either of the other types. Indeed, there are often several hundred times as many ovaries as spermaries and in some cases the worm passes through a strictly female phase. In other individuals there may be a few spermaries at either end of the body or they may be scattered between the ovaries. It must be remembered, however, that this condition applies to the individual at the time of observation or the time of collection and does not indicate the sexuality which has obtained at an earlier period of life or might have followed later if the animal had survived.

The hermaphroditic gonad may occasionally function first as a spermary and later as an ovary but more often the ovocytes present undergo cytolysis before the sperm are fully mature. Otherwise each gonad functions but once, its place being taken by a younger one following the discharge of the gametes, or of the embryo in the viviparous species, as *G. agricola*. New gonads are formed throughout life from groups of germinal cells situated along the dorsal borders of the nerve cords (Figs. 4, 5). It is conceivable that the sexual differentiation of the gonad may depend upon the nutritive conditions of that region of the body at the time of differentiation. Presumably when closely crowded or when originating in an unusual position on the distal side of the nerve cord, as well as when rapidly growing ovocytes or embryos are present nearby, the gonad becomes differentiated into a spermary. When a spermary develops in the same interdiverticular space as an ovary the former usually lies nearest the nerve cord.

Fertilization

In nearly all the species in which living individuals have been available for study the worms have been observed to collect together in groups with the bodies of two or more individuals placed side by side, accompanied by the secretion of considerable mucus. If two or more of these individuals are provided with ripe sexual products when thus in contact the sperm cells from one individual may be readily transferred to the fertilization chambers of another which may contain

mature ova and open oviducts. Internal insemination occurs in all terrestrial species so far as known but is not confined to that group, since it is also characteristic of the fresh-water nemerteans and some of the marine species.

In *G. australiensis*, in which the sexes are separate, such copulation and the transfer of sperm from the small male to the larger female has been observed (Dendy, 1893). The sperm thus transferred to the ovaries may remain functional for several days or perhaps weeks, ready to enter the egg in each ovary as soon as it has become sufficiently mature. Although the male in *C. dendyi* has not yet been discovered, Waterston and Quick (1937) reported that several of the females collected by them laid clusters of fertilized eggs a few days after capture.

In the hermaphroditic species the young, male-phase individual presumably may thus transfer his sperm to either an individual functioning at the time as a female or to an hermaphrodite with both ripe eggs and sperm; likewise two hermaphrodites may transfer sperm mutually or an hermaphrodite may inseminate a functional female. It seems quite possible that in some cases individuals of all these sexual phases may be grouped together at the same time. Microscopic sections sometimes show the presence of several spermatozoa in a fertilization chamber.

In terrestrial nemerteans insemination thus precedes ovulation if two individuals with suitable sexual products come in contact and this is the case with a few marine and fresh-water forms. In the great majority of nemerteans, however, the eggs are fertilized at the time of ovulation or in some cases, as in *Cerebratulus*, vast numbers of minute ova are set free in the water with the possibility of fertilization by sperm originating from a male which may be at least several meters distant.

There appears to be no fixed limit to the number of sexual phases which an individual may experience during its lifetime, since even in the largest worms bearing ripe ova or embryos, according to the species, there are always present many newly-formed, undifferentiated gonads in the parenchyma adjacent to the dorsal sides of the nerve cords (Figs. 1, 4). Recuperation periods, some of which may be of a seasonal nature, may, presumably, long delay the completion of any of these phases.

Self-fertilization

In the hermaphroditic species, as *G. agricola* and *G. palaensis*, spermaries with fully functional sperm are frequently present in some part of the body and sometimes immediately adjacent at the time when the ova are ready for fertilization. It would appear that in isolated individuals the conditions are highly favorable for self-fertilization, although direct evidence of such an occurrence is not available. It is well-

known, however, that in some hermaphroditic nemerteans, as the fresh-water *Prostoma*, self-fertilization takes place frequently.

It is remarkable that among the 12 known species of terrestrial nemerteans, all of which belong to a single genus, there should be so much similarity in morphological and ecological characteristics and so great a diversity in the physiology of reproduction. Some of the species are hermaphroditic and others presumably of separate sexes; most of them are known or believed to be oviparous but at least one species is regularly viviparous.

Viviparity, protandry and hermaphroditism occur also in a few species of marine nemerteans belonging to different groups, and in some species of *Prostoma*, *Carcinonemertes* and *Prosorhochmus* which are normally oviparous, with internal fertilization, a few of the fertilized ova may be retained in the mother's body until the embryo is far advanced. Since in *Geonemertes* the same type of fertilization exists and at least one of the species is regularly viviparous it seems quite possible that occasional viviparity will later be found in some of the little-known species now thought to be exclusively oviparous.

SUMMARY

1. A series of 21 specimens of a species of terrestrial nemertean, *Geonemertes palaensis*, from tropical Pacific islands includes successive stages in the development of the gonads which show that this species is normally protandric and irregularly hermaphroditic, as previously described for *G. agricola*.

2. The primary male phase of the young individual is followed by a female phase which, however, may become functionally hermaphroditic in some individuals before the ova are fully ripe by the precocious development of some of the spermaries of the normally succeeding male phase. Opportunity is thereby offered for self-fertilization.

3. The extent to which the female phase toward its close is accompanied by the development of spermaries is highly variable, some individuals having in this phase only a few small spermaries and others many larger ones.

4. This sequence of alternating male and female (or hermaphroditic) sexual phases presumably continues throughout the lifetime of the individual.

5. *Geonemertes palaensis* is oviparous, while the terrestrial nemertean of Bermuda (*G. agricola*) is viviparous. The sexual phases, however, are similar in both species.

6. No direct evidence is available as to whether the great variability in the relative proportions of male and female gonads in different indi-

viduals following the initial male phase in both these species is due to physiological conditions imposed by environmental influences or to slight differences in the hereditary sexual endowment. Presumably both these factors may be associated.

LITERATURE CITED

- BÖHMIG, L., 1898. Beiträge zur Anatomie und Histologie der Nemertinen. *Zeitschr. f. wiss. Zool.*, **64**: 479-564.
- COE, W. R., 1904. The anatomy and development of the terrestrial nemertean (*Geonemertes agricola*) of Bermuda. *Proc. Boston Soc. Nat. Hist.*, **31**: 531-570.
- COE, W. R., 1904a. Sexual phases in *Geonemertes*. *Zool. Anz.*, **28**: 454-458.
- DAKIN, W. J., 1915. Fauna of West Australia. III. A new nemertean *Geonemertes dendyi*, sp. n. being the first recorded land nemertean from Western Australia. *Proc. Zool. Soc. London*, pp. 567-570.
- DENDY, A., 1893. Notes on the mode of reproduction of *Geonemertes australiensis*. *Proc. Roy. Soc. Vict.*, **5**: 127-130.
- DENDY, A., 1895. Notes on a New Zealand land nemertine. *Trans. and Proc. New Zealand Inst. (Wellington)*, **27**: 191-194.
- DENDY, A., 1896. Note on the discovery of living specimens of *Geonemertes novae-zealandiae*. *Trans. and Proc. New Zealand Inst. (Wellington)*, **28**: 214-215.
- GRAFF, L., 1879. *Geonemertes chalicophora*, eine neue Landnemertine. *Morph. Jahrb.*, **5**: 430-449.
- HETT, M. L., 1924. On a new land nemertean from New South Wales (*Geonemertes hillii* sp. n.). *Proc. Zool. Soc. London*, pp. 775-787.
- HETT, M. L., 1928. On some land nemerteans from Upolu Island (Samoa) with notes on the genus *Geonemertes*. *Proc. Zool. Soc. London*, 1927, pp. 987-997.
- KENNEL, J. VON, 1878. Beiträge zur Kenntniss der Nemertinen. *Arb. Zool.-soot. Inst. Würzburg*, **4**: 305-381.
- SCHRÖDER, O., 1918. Beiträge zur Kenntniss von *Geonemertes palaensis* Semper. *Senkenburg. Natur. Gesellschaft*, **35**: 153-177.
- STAMMER, H.-J., 1934. Eine für Deutschland neue eingeschleppte Landnemertine, *Geonemertes dendyi* Dakin, mit einer Bestimmungstabelle der Gattung *Geonemertes*. *Zool. Anz.*, **106**: 305-311.
- WATERSTON, A. R., AND H. E. QUICK, 1937. *Geonemertes dendyi* Dakin, a land nemertean, in Wales. *Proc. Roy. Soc. Edinburgh*, **57**: 379-384.

MORTALITY OF THE COD EGG IN RELATION TO TEMPERATURE

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The general fact that the rate of development of fish eggs becomes greater as the temperature is increased is well known, but knowledge of the relative mortality of the eggs at different temperatures is poor. In nature, the extent of mortality of the egg is particularly important in the case of marine fish which produce pelagic eggs because such eggs may be carried by currents from the spawning grounds into regions of different temperature. The location in which the majority of the eggs will ultimately hatch depends not only upon the rate of development but more upon the relative mortality at various temperatures encountered. (Cf. Bigelow, 1926; Fish, 1927.) Conversely, in cases where developing eggs or larvae are taken in plankton hauls, the site at which the eggs were spawned may be traced provided the rates of development and mortality are known and the velocity and direction of the current and temperature of the water can be ascertained. (Cf. Walford, 1938; Jacobsen and Johansen, 1908.)

The experiments described in this paper were undertaken to test the extent of mortality in the cod egg at various temperatures and to determine the highest temperature at which development is possible for this species.

Dannevig (1894), who made the first comprehensive attempt to find the temperature range for the cod, was able to hatch eggs from 0° to 14° C. Johansen and Krogh (1914), the most recent investigators in this field, have confirmed this pioneer work. Although chiefly interested in the temperature coefficient, they carried out experiments to establish the upper temperature limit, which they placed at 10.2° C., as eggs hatched at this temperature but failed to do so at 12°, 13°, 16.5° and 20° C.

Investigators who have been more directly concerned with mortality in fish development are Drouin de Bouville (1908), Hein (1907, 1911), Hata (1927), and Rollefson (1932). Hein subjected trout eggs to adverse conditions at various times after fertilization and demonstrated

¹ The author wishes gratefully to acknowledge the advice and encouragement of Dr. George L. Clarke of Harvard University.

that variations in mortality occurred in relation to the length of time development had proceeded. Rollefson has confirmed these results, particularly for the early stages of development, and has correlated the decrease in susceptibility to mechanical shock, with the closing of the blastopore.

PROCEDURE

The cod egg was chosen for these experiments because it normally floats at the surface of the water while alive and sinks when dead or dying, thus presenting a clear-cut criterion of death. Since the chorion is transparent, an unhampered view of the embryo is afforded and consequently the stage of development may be easily determined. Material was readily obtained through the courtesy of the Gloucester Station of the United States Bureau of Fisheries.

Eggs were obtained from cod taken in Ipswich Bay, Massachusetts, on March 31, 1937. The surface temperature was 2.5° C. The eggs were transported in insulated jugs at 2.5–3° C., to the laboratory where they were apportioned into beakers at the different constant temperatures. Eight hours had elapsed since fertilization but due to the low temperature the eggs were only just past first cleavage. (Plate I, Fig. 2.)

The 500-cc. beakers used were open at the top, and only 200 cc. of aerated sea water were introduced in order to provide a relatively large surface area for gas exchange. The beakers were placed on racks in five constant temperature tanks maintained to within 0.3° C. at 6, 8, 10, 12, and 14° C. respectively, by means of thermo-regulators. The first four tanks were placed in a cold room at 2° C. and each appropriately heated. The fifth tank was kept in a laboratory at room temperature and cooled to the desired temperature by means of a Kelvinator refrigerating unit.

The sea water, which had a salinity of 32.31‰, had been obtained at Nahant, Massachusetts, six months previously and allowed to stand. During this period all organic matter had decomposed and fouling of the water during the experiment was reduced to a minimum. Although this decomposition process reduced the oxygen content so that thorough aeration of the sea water was necessary before use, it was not necessary to aerate the jars containing the eggs during the experiment.

The eggs were distributed among twenty beakers and four beakers were placed in each of the five tanks. Each day the eggs were transferred to fresh beakers containing water that had been prepared in advance and adjusted to the proper temperature. Transfer was accomplished by means of a scoop made of fine mesh nickel screening.

The eggs which sank to the bottom—and hence were judged to be dead—were removed daily from each jar by pipette, counted and preserved. The fixative used was Stockard's solution² which turns the embryo white and leaves the yolk clear. This facilitates the determination of the degree of development in preserved material.

In many cases the eggs were examined before preservation and there was no doubt that they were either dead or dying.

The live eggs in two of the beakers (jars *A* and *B*) in each tank were sampled daily in small numbers to determine the stage of development. They were examined before preservation and the stage carefully noted according to the plan presented in Plates I and II. In every sample the eggs were all found to be very near the same stage. In a few cases in which the stage of a small number departed noticeably from that of the majority, the embryos were obviously monsters or aborted forms whose development could not have proceeded much further. These abnormal forms were encountered more frequently during the early days of development than later.

This daily sampling of jars *A* and *B* resulted in a continued reduction of the total numbers of eggs. Since it was feared that this change in concentration might influence the mortality, parallel tests without sampling were made in the third and fourth beakers in each tank (jars *MA* and *MB*). Only dead eggs were removed in the case of these two beakers. However, the mortalities which resulted in the jars that were sampled turned out to be substantially the same as those obtained for jars that were not sampled.

RATE OF DEVELOPMENT AND DRIFT AT SEA

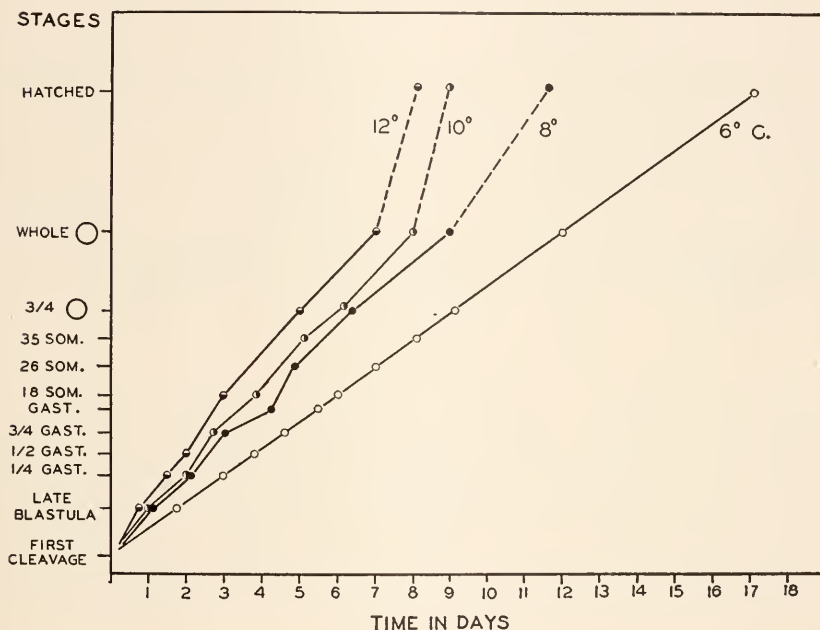
The considerable lapse in time between spawning and hatching for the various temperatures tested may be appreciated from the curves plotted in Fig. 1. The tests run at 14° showed no survival after 24 hours and a subsequent test at 13° also failed. Hence, 12° C. was concluded to be very near the upper limit of temperature for the development of the cod egg. The low survival at this temperature and previous work of Dannevig (1894) and Johansen and Krogh (1914) are in accord with this view.

The young fry of the cod are believed to take to the bottom 7 days after hatching according to McIntosh and Masterman (1897). It is of interest to calculate the point to which Ipswich Bay cod eggs will drift and to ascertain whether the young will find themselves in a suitable locality when they are ready to take to the bottom. According to Fish

² Stockard's solution consists of 5 parts 40 per cent formalin, 6 parts glycerin, 4 parts glacial acetic acid, and 85 parts distilled water.

(1927) most of the eggs produced in Ipswich Bay do not drift into Massachusetts Bay but are carried off to the southward. These eggs may be supposed to drift at a rate similar to those leaving Massachusetts Bay, which were shown by Fish to move about 3 or 4 miles per day. At 6° development to hatching required 17 days. With 7 days added to reach the bottom, the eggs would drift 72 to 96 miles.

It is probable that the temperature at which the Ipswich Bay eggs develop is considerably lower than this. Bigelow and Welsh (1925)



TEXT FIG. 1. The development of the cod egg at four different constant temperatures. Abscissa: Time in days. Ordinate: Stage of development determined by the 6° C. which is arbitrarily plotted as a straight line. The curves for the other temperatures plotted against the stages so determined.

state: "The chief production of cod eggs is during the cold months, on the Ipswich grounds, for example, ripe fish are taken when the bottom water is still as warm as 6.6 to 7.7° C. in early September, but they appear in great numbers in temperatures of 5° to 6° C. in January, and as the breeding progresses, the temperature falls, spawning being at its height in the minimum temperature of the year (March), that is at 0.5° to 3.0° C." Poulsen (1931), McKenzie (1934), and others agree that cod may spawn at temperatures ranging from 8° down to 0° C. At a temperature of 3° C. hatching occurs in 23 days according to

Dannevig (1894). With 7 days added for larvae to take to the bottom, at a maximum of 4 miles per day the egg would travel 120 miles from its spawning ground. In the present case such a journey would carry the egg to the northwestern edge of Georges Bank. This accords well with Fish (1927) that the eggs spawned in inshore areas such as Ipswich Bay take to the bottom on Georges Bank.

We may next inquire whether the temperature in this region during the critical months ever becomes sufficiently high to result in serious mortality to the cod eggs. Bigelow (1927) has shown for the Gulf of Maine that the temperatures which prevail during the spring months and early summer are well below 12° C. During February and March the highest surface temperature recorded was 5° C., while during April, the temperature at the surface was below 6° C. In May, the waters have warmed to 10° C. in a few places, but the temperature does not rise above 12° C. until the latter part of July and during August. Inasmuch as this is well after the eggs have hatched, mortality due to too high temperatures is not normally a factor limiting the distribution of the developing cod egg from the Ipswich Bay spawning grounds.

OBSERVATIONS ON MORTALITY

Sources of Error

The principal source of error in this experiment was the use of the sinking of the egg as a criterion of death. On several occasions it was noted in sampling the floating eggs that there were some eggs, particularly among the early stages, which were only masses of cells without

Stages in the development of the cod. Magnification 30 ×. Figures marked with (*) were used as stages of development.

FIG. 1. Protoplasmic cap, prior to first cleavage. Drawn from material preserved 15 minutes after fertilization.

FIG. 2*. Two-cell stage. Ten hours after fertilization at 0°–3° C. Stage at distribution of eggs.

FIG. 3. Eight-cell stage, polar view. Twenty-eight hours after fertilization at 0°–3° C.

FIG. 4. Early blastula, 52 hours after fertilization at 0°–3° C.

FIG. 5. Middle blastula. Seventy-six hours after fertilization at 0°–3° C. Twenty-four hours after Fig. 2 at 6° C.

FIG. 6*. Late blastula. One hundred hours after fertilization at 0° C. Twenty-four hours after Fig. 2 at 10° C.

FIG. 7. Early gastrula. Forty-eight hours after Fig. 2 at 6° C.

FIG. 7a. Ibid. Polar view.

FIG. 8*. One-quarter gastrula. Fifty-two hours after Fig. 2 at 6° C.

FIG. 9*. One-half gastrula. Seventy-three hours after Fig. 2 at 6° C.

FIG. 9a. Ibid. Eccentric polar view.

FIG. 10*. Embryo beyond the three-quarters gastrulation stage; 120 hours after Fig. 2 at 6° C. First specks of pigment appearing.

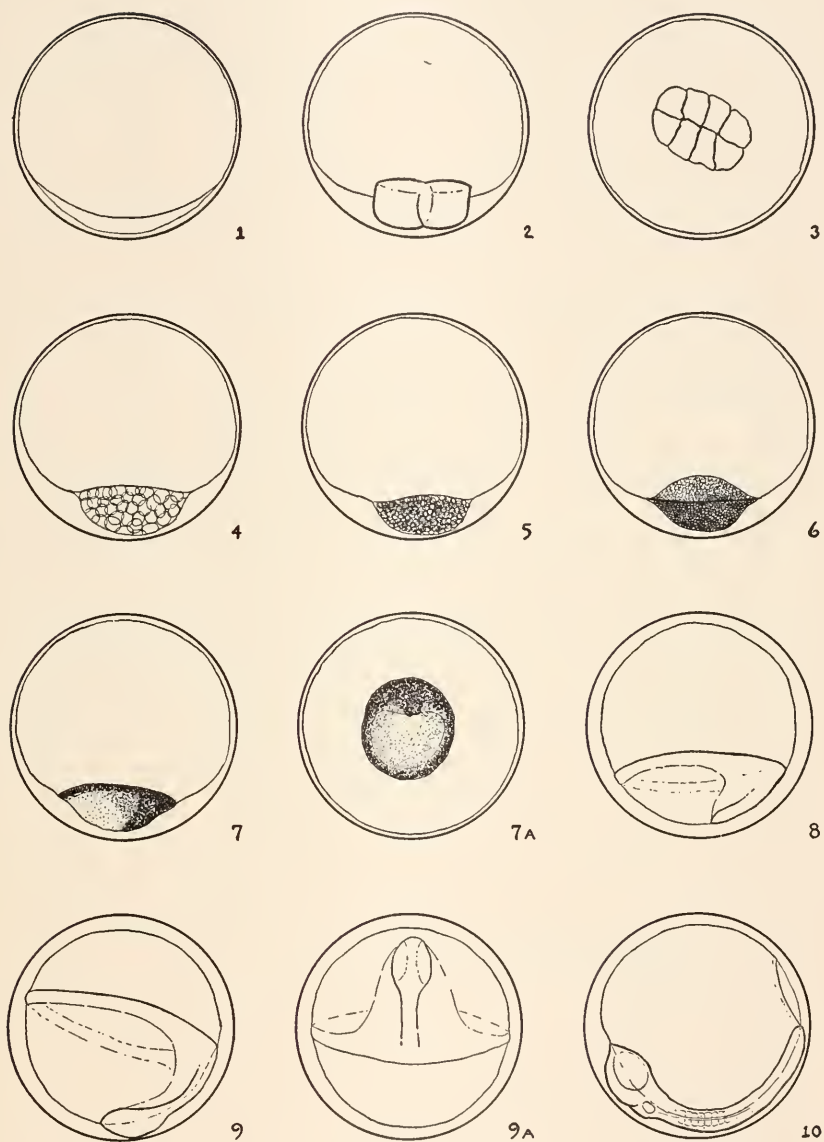


PLATE I

any morphological features to determine the stage. Brice (1898) states that floating eggs are not necessarily alive, for unfertilized and injured eggs usually float for 18 to 36 hours before going to the bottom. In dealing with daily mortality, one must recognize the possibility of a lag of this duration. Observations on such aborted eggs indicated that the number at any time was negligible in relation to the total number which sank during 24 hours.

That some eggs which sink may be alive is likewise a possibility. In fact, McIntosh (1893) states that in certain of his experiments with the cod, the eggs retained their vitality although they did not float. But in the present experiment, examination of the non-floating eggs showed that less than 1 per cent appeared normal. Furthermore, it was observed that death soon followed when the eggs sank, although development did continue for a short time.

During the later stages of development this criterion becomes less certain, for Jacobsen and Johansen (1908) have shown that the specific gravity of the egg becomes greater as development proceeds. It was observed that at 6° C. all the eggs floated at the surface until the tenth day after which the eggs became scattered in various layers in the water. This increased the difficulty of removing only dead eggs. Therefore from the tenth day opaqueness of the egg was used as the criterion of death. Since only about 2 per cent of eggs removed contained embryos with beating hearts, important numbers of live eggs were not being removed with the dead eggs. Although a certain number of dead eggs which had not yet become opaque were probably missed, the effect

Stages in the development of the cod. Magnification 30 ×. Figures marked with (*) were used as stages of development for graphs.

FIG. 11*. Just prior to whole gastrula or final closing of the blastopore; 126 hours after two-cell stage at 6° C. (Plate I, Fig. 2.) Embryo of 11 somites. Apolar view.

FIG. 12*. Embryo of 18 somites; 144 hours after two-cell stage at 6° C. Pigment definitely appearing.

FIG. 12a. Ibid. Apolar view. Pectoral limb buds forming.

FIG. 13*. Embryo of 35 somites. Tail twisting to the right; 192 hours after two-cell stage at 6° C.

FIG. 14*. Three-quarters circle. Apolar view. Heart beginning to beat. Muscular movements of the tail; 216 hours after two-cell stage at 6° C.

FIG. 14a. Ibid. Lateral view.

FIG. 15*. Whole circle. Tail approximates head. Upper part of eye well pigmented; 288 hours after two-cell stage at 6° C.

FIG. 16. Just prior to hatching. Pigment localized into definite bands. Eye well pigmented; 396 hours after the two-cell stage at 6° C.

FIG. 17. Lateral view of head of Fig. 16.

FIG. 18. Larval cod about three days after hatching. Floats with yolk-sac uppermost during first two days after hatching at 6° C.

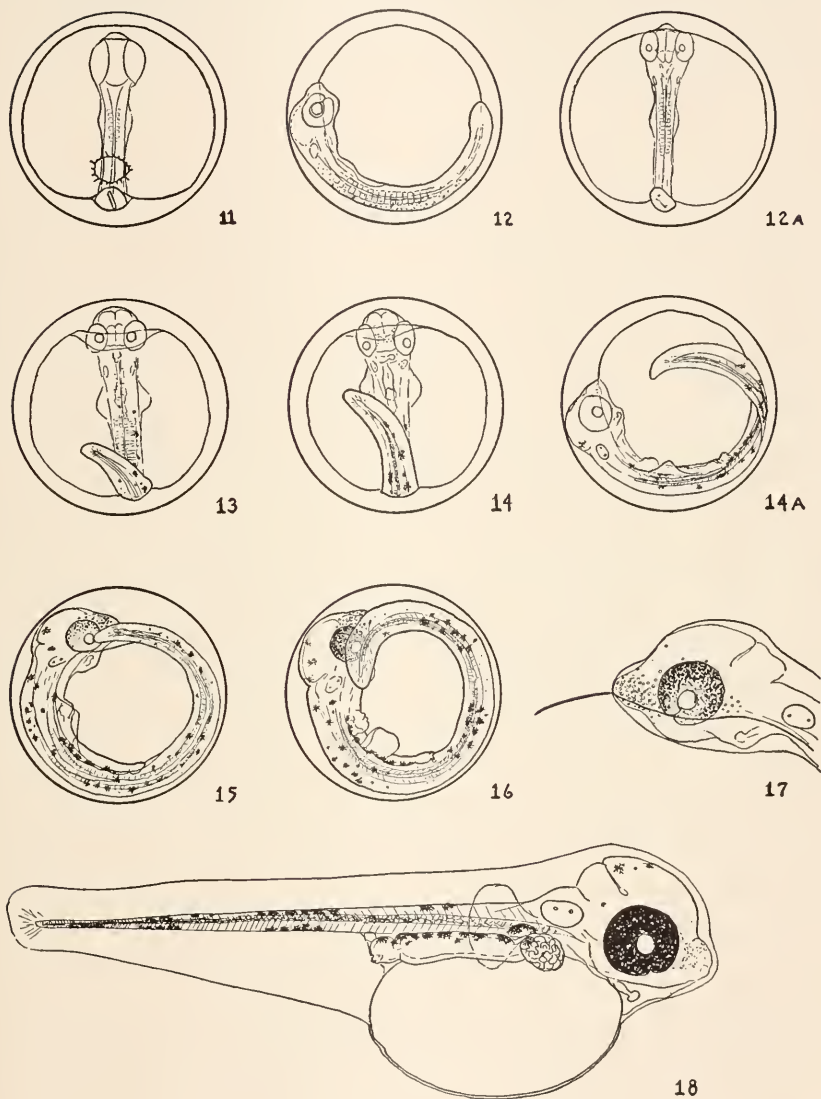


PLATE II

of this procedure merely introduced a delay in the time when those eggs would be counted as dead. The high mortality which occurred in these experiments does not interfere with an interpretation of the results, for the interest lies not in the number of deaths but in the distribution of mortality in relation to the stage of development.

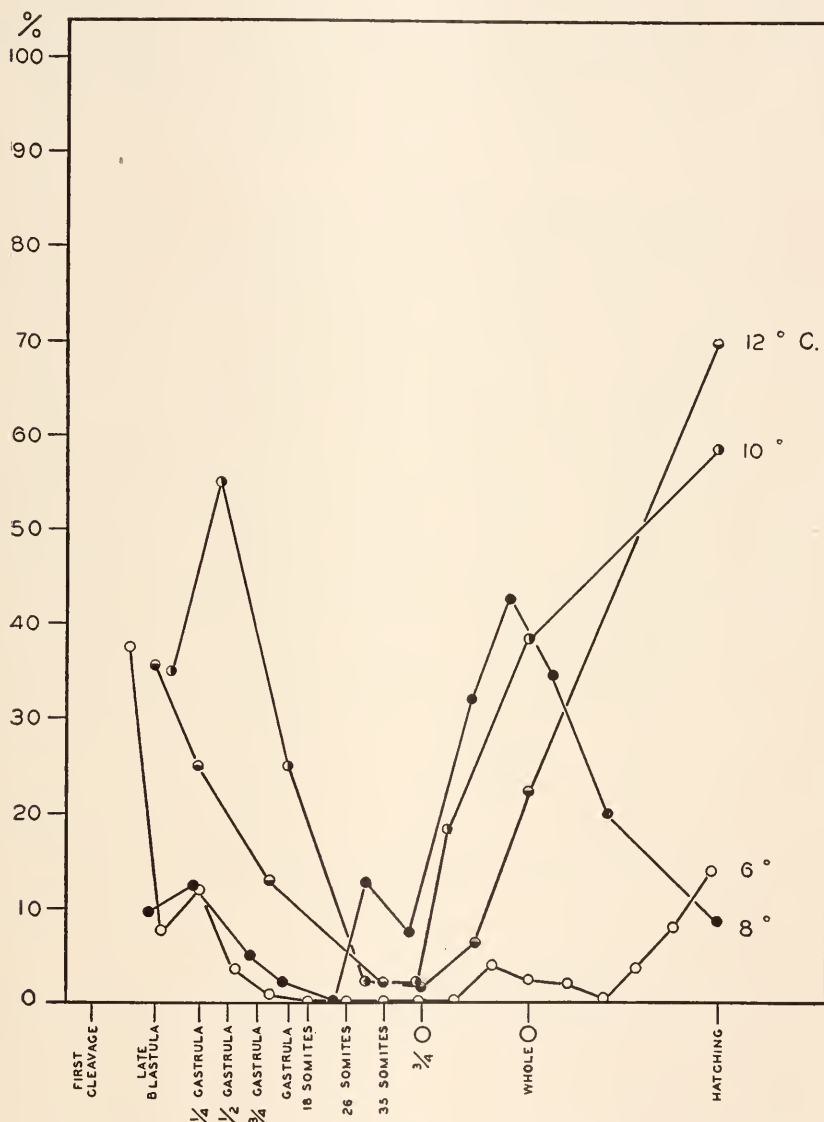
Results of Experiments

The time required for the eggs to reach various stages at different temperatures may be observed in Fig. 1, in which the data obtained from daily sampling have been plotted following the method used by Worley (1933) for the mackerel. Those stages in development of the cod which have been used for this purpose are indicated in the legends of Plates I and II. The stages plotted are not of equal duration, as they were chosen on the basis of certainty of recognition. They have been spaced on the graph in such a way as to make the 6° C. development approximate a straight line. The development of the eggs at the other temperatures was then plotted against these same ordinates.

Confusion has existed in the past over the use of "hatching" as a stage in development because the times when the first and last egg hatch are ordinarily observed directly whereas in the case of the other stages, the samples of eggs withdrawn for microscopic examination allow only the modes of the stage to be determined. Obviously, if the time for each of the earlier stages is taken as the mode for the whole population, then the time designated for the hatching stage should be the point at which the largest number of eggs hatch. Merriman (1935), in his work on the cut-throat trout, however, has taken the time to "first hatching" as one stage and the "eyed-out" condition as another stage. This procedure is fallacious unless the moment when the first embryo entered the eyed-out stage was observed. But even if this were done, the method gives a poorer measure of the rate of development for the whole population than the use of the mode.

Merriman objects to employing the middle of the hatching period as the time to "hatching" because he finds that the "*duration*" of the hatching period (i.e. the time from the hatching of the first egg to the hatching of the last) varies with the temperature in a different manner than the duration of the whole period of development. But the amount of "spread" of the eggs in any stage has no necessary connection with the changes in average time for complete development at different temperatures. Furthermore, in stages other than "hatching" the time that each egg remains in a given stage may be affected differently by temperature at the various points of development. The influence of tem-

perature on the total time for development, and on the time elapsed in each stage should therefore be carefully distinguished. In the present case the information desired is the average total time for development



TEXT FIG. 2. The percentage mortality of the cod egg in relation to stage of development. Abscissa: Stage of development. Ordinate: Percentage daily mortality. (The number of eggs that died during any day divided by the number of eggs that were alive at the beginning of that day multiplied by 100.)

involving a summation of the average times in each of several stages. Therefore, the time taken should be the time required for the "modal" or typical egg at each temperature. Worley (1933) accomplished this by means of frequency distribution. In the present experiment, the number hatching during each day was observed. The time of "hatching" was taken as the day when 50 per cent of all the eggs that hatched during the entire hatching period had completed their development in

TABLE I

Percentage of eggs hatching at different constant temperatures

Temperature	Jar	Total no. of eggs	Number hatched	Percentage hatched of total eggs	Average
° C.				<i>per cent</i>	<i>per cent</i>
6	IA	1653	10	0.6	11.2*
	IB	2059	8	0.4	
	IMA	118	20	16.9*	
	IMB	546	148	27.1*	
8	IIA	1913	84	4.3	3.3
	IIB	2975	124	4.1	
	IIIMA	1811	70	3.8	
	IIMB	1509	15	1.0	
10	IIIA	1820	60	3.2	1.9
	IIIB	339	7	2.0	
	IIIMA	1638	6	0.3	
	IIIMB	1730	37	2.1	
12	IVA	2831	2	0.07	0.04
	IVB	2837	0	0.0	
	IVMA	2312	0	0.0	
	IVMB	1690	2	0.12	

* These values are not comparable to the other figures since the initial number of eggs is very low and the factor of crowding must be considered.

the egg stage,—a method not quite as accurate but more convenient in the case of a slowly developing egg.

A comparison of the curves of Fig. 1 reveals the fact that when the stages are so arranged that a straight line results at 6° C., approximately straight lines are obtained for the other temperatures. This means that the effect of different temperatures on the rate of development of the modal egg was relatively the same in all stages. At 6° C. development was almost twice as long as the development at 12°. This compares favorably with the results obtained by Dannevig (1894) and Johansen and Krogh (1914). The difference between 10° and 12° is much less

than that between 6° and 8° although the temperature change in both cases is 2° C. The explanation lies in the fact that we are dealing with the upper part of the temperature range, the optimal temperature being about 6.5° C., and as the maximum is approached there is a retardation of development. Worley (1933) has demonstrated a similar situation in the mackerel.

The percentage mortality at the different temperatures has been plotted against the stage of development in Fig. 2. The percentage mortality is obtained by dividing the total number of live eggs at the beginning of any 24-hour period into the number of eggs that died in the same period. During the hatching period all eggs which had hatched on previous days were subtracted from the total. The stages of development were interpolated from Fig. 1, and the curves presented here are the mean values for all the jars in each tank. In order to determine accurately the total number of eggs in each jar at the beginning of the experiment, the number of eggs that died daily and the number that hatched were added together. These totals are tabulated in Table I.

Special mention must be made of the 8° C. curve. The first batch of eggs all died on the first day due to a failure in the apparatus which caused a sudden rise in temperature. A second lot of eggs which had been held at 2° C. for 24 hours before being placed at 8° C. was used. At this time, they were at a stage corresponding to early blastula (Plate I, Fig. 4). The wide variation which occurred day by day in the various jars in the 8° C. tank seems to indicate an extraneous factor influencing the mortality. The consistency between the individual jars would indicate a factor common to them all. In view of the fact that the curves for the other tanks are consistent among themselves, one should view the curve of the 8° tank with reservations in considering the general implications of this experiment.

An examination of the curves in Fig. 2 of the percentage mortality at 6, 10, and 12° C. shows that there is general agreement in two respects: a decided decrease in mortality during closing of the blastopore and an increase in mortality as hatching is approached. A different way of stating this conclusion is that the susceptibility of the egg to harmful factors in the environment is least between closure of the blastopore and the first signs of muscular movement, which occurs when the embryo occupies approximately three-quarters of the circumference of the egg (Plate II, Fig. 14). The curve for 8° C. shows the same general trend but the large fluctuations appear to have no satisfactory explanation. The important feature to be noted in these curves is their similarity. There is a decline in mortality toward the same stage irrespective of the temperature. To be sure, the length of the period of

low mortality is longest at 6° C., which is near the optimum temperature, but the periods of high mortality are the same for 6° as for the other temperatures. Since greater numbers of eggs die at 12° C., the higher average position of the curve is understandable.

Rollefsen (1932) has shown that a decrease in susceptibility of cod eggs to shock caused by dropping occurred coincident with the closing of the blastopore. This he explained as an increase in the strength of the yolk covering, preventing rupture. Hein (1907, 1911) performed a series of experiments on trout eggs, subjecting them to various types of adverse conditions at different times after fertilization, and found a similar decrease in susceptibility down to closure of the blastopore and an increase just prior to hatching. My results have confirmed these earlier experiments, but it is not possible at the present state of our knowledge to make any statement concerning correlations between susceptibility and ontogenetic stage beyond the gross description of coincident states.

SUMMARY

1. Experiments were carried out at four different temperatures in the supra-optimal range of development for the cod egg.

2. Development from the two-cell stage to 50 per cent hatched required 8.5 days at 12° C., 9 days at 10° C., 11.5 days at 8° C., and 17.2 days at 6° C.

3. At all temperatures an initial period of high mortality decreased with the closing of the blastopore and was followed by a period of low mortality until the embryo was three-quarters the circumference of the egg membranes, when the mortality steadily increased up to hatching.

4. The hatching period is discussed with reference to its use as a stage of development.

BIBLIOGRAPHY

- BIGELOW, H. B., 1926. Plankton of the offshore waters of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40** (Part II): 1-509.
- BIGELOW, H. B., 1927. Physical oceanography of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40** (Part II): 551-1027.
- BIGELOW, H. B., AND W. W. WELSH, 1925. Fishes of the Gulf of Maine. *Ibid.*, **40** (Part I): 1-565.
- BRICE, J. J., 1898. A manual of fish culture. *Report Comm. Fish and Fisheries, U. S., 1897 (1898)*, Part XXIII appendix.
- DANNEVIG, A. 1894. The influence of temperature on the development of the eggs of fishes. *Thirteenth Ann. Report Fish. Bd., Scotland*, pp. 147-152.
- DROUIN DE BOUVILLE, R., 1908. Influence des variations thermiques brusques sur les oeufs, alevins et jeunes sujets de salmonides. *Compt. Rend. Soc. de Biol.*, **65**: 259-261.
- FISH, C. J., 1927. Production and distribution of cod eggs in Massachusetts Bay in 1924 and 1925. *Bull. U. S. Bur. Fish.*, **43** (Part II): 253-296.

- HATA, K., 1927. On the influence of four kinds of vibration upon the eggs of *Onchorhynchus masou* Brevoort. *Jour. Imper. Fish. Instit. Tokyo*, **23**: 74-78.
- HEIN, W., 1907. Zur Biologie der Forellen Brut. III. Ueber die Wirkung von Druck, Stozz und Fall auf die Entwicklung der Bachforelleneier. *Allgemeine Fischerzeitung*, **32** (18): 383-387.
- HEIN, W., 1911. Ueber die Wirkungen plotzlicher Temperaturschwankungen auf die Eier und Brut von Bachforellen. *Ibid.*, **36** (23): 505-510.
- JACOBSEN, J., AND A. C. JOHANSEN, 1908. Remarks on the changes in specific gravity of pelagic fish eggs and the transport of same in Danish waters. *Medd. fra Komm. f. Havundersogl., Series Fiskeri*, **3**: 1-24.
- JOHANSEN, A. C., AND A. KROGH, 1914. The influence of temperature and certain other factors upon the rate of development of the eggs of fishes. *Conseil perm. internat. pour l'explor. de la mer, Publ. de Circonstances*, No. 68.
- MACINTOSH, W. C., 1893-1894. Contributions to the life histories and development of the food and other fishes. *Eleventh and Twelfth Ann. Reports Fish. Bd. Scotland*, pp. 239-248, 218-230.
- MACINTOSH, W. C., AND A. T. MASTERMAN, 1897. British Marine Fishes. First Edition. C. J. Clay and Sons. London.
- McKENZIE, R. A., 1934. Cod and water temperatures. *Progress Report Atlantic Biol. Sta. Canada*, Note 41, pp. 3-6.
- MERRIMAN, D., 1935. The effect of temperature on the development of the eggs and larvae of the cut-throat trout (*Salmo clarkii* Richardson). *Jour. Exper. Biol.*, **12**: 297-305.
- POULSEN, E. M., 1931. Biological investigations upon the cod in Danish waters. *Medd. fra Komm. f. Denmark. Fisk. og. Havun. Series Fisk*, **9** (1): 1-148.
- ROLLEFSEN, G., 1932. The susceptibility of cod eggs to external influences. *Jour. de Conseil perm. internat. pour l'explor. de la mer*, **7** (3): 367-373.
- WALFORD, L. A., 1938. Effect of currents on the distribution and survival of the eggs and larvae of the haddock (*Melanogrammus aeglefinus*) on Georges Bank. *Bull. U. S. Bur. Fish.*, **49** (29): 1-73.
- WORLEY, L. G., 1933. Development of the eggs of the mackerel at different temperatures. *Jour. Gen. Physiol.*, **16** (5): 841-857.

THE TIME OF EMBRYONIC DETERMINATION OF
SENSORIA AND ANTENNAL COLOR AND
THEIR RELATION TO THE DETER-
MINATION OF WINGS, OCELLI,
AND WING MUSCLE IN
APHIDS ¹

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In a general analysis of the applicability of the time-of-determination theory to intermediate aphids by Stiles (1938), nothing was said regarding the color of antennae and number of sensoria except to include them in the same tables with the wing, ocellar, and wing muscle development. The purpose of this paper is to report a study of the time of embryonic segregation of the sensoria and antennal color in the aphids previously reported (Stiles, 1938).

Shull, in investigations of gamic-parthenogenetic (1933) and winged-wingless (1937) intermediate aphids, did not include individuals whose intermediacy consisted in the color of their antennae because of the apparently erratic variability of that color. Nevertheless, antennal color and sensoria number are both characters very definitely related to the winged condition of aphids, hence should receive consideration in a study of forms intermediate between winged and wingless. Because most of the intermediates of this study did not express any intermediacy in their antennae, it was difficult to relate closely the intermediacy of this organ with that of the wings, ocelli and wing muscle. No closely graded series of antennal color or sensoria number could be found; therefore, it became apparent that these characters were probably of less value for this type of investigation than the wings, ocelli and wing muscle. It is for this reason that the records are somewhat incomplete regarding the nature of the antennae in the data presented (Stiles, 1938). However, enough antennae were studied to make possible an analysis of their relation to the general problem of intermediacy.

In my previous paper the antennae of the typical wingless aphid with respect to color are rated 0 (light) and that of the winged as 4 (dark), with intermediates represented by numbers between these. While 4 to 6 sensoria in the third segment of the antenna are typical

¹ Contribution from the Department of Zoölogy, University of Michigan.

in the wingless (Fig. 1 *A*) and about 15–18 in the winged aphids (Fig. 1 *B*), it is not considered safe, due to variation, to judge less than 10 sensoria as representing certain intermediacy in an antenna, although there are doubtless some with less that should be so considered.

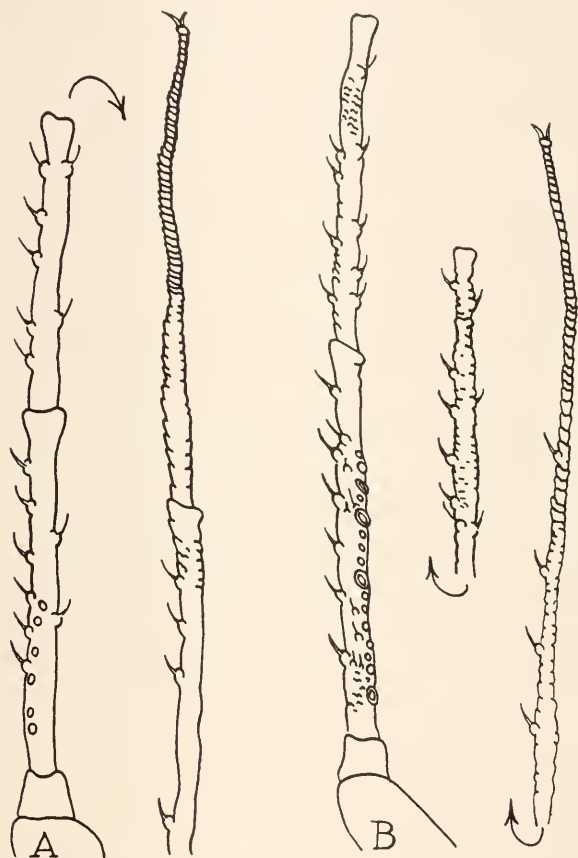


FIG. 1. Antennae of *Macrosiphum solanifolii* showing the difference in number of sensoria in typical wingless and winged forms. Sensoria are represented by the small circles on segment III. *A*, Wingless parthenogenetic female. *B*, Winged parthenogenetic female.

The data presented in Tables ² I to IX inclusive (Stiles, 1938) show that the color of antennae and sensoria number are closely related; that is, in general a darkening of antennae is accompanied by an increase in sensoria number. The author is inclined to believe that, in a case

² To save republication of tables, all tables cited in this paper refer to a previous paper (Stiles, 1938).

where a slight darkening of the antennae may have been recorded with no corresponding increase in the number of sensoria, an error has been made in judging antennal color. Unless the color difference is marked, it is difficult to be sure of the antennal color. Nevertheless, records of both antennal color and sensoria number are useful inasmuch as one provides a check on the other. Since there has been found to be a close correlation between the number of sensoria and antennal color, with the number of sensoria a more reliable guide to antennal intermediacy than the color of antennae, henceforth these structures will be indicated by statements concerning only the sensoria.

With few exceptions, the data showed a correlation between the sensoria and the character of the wing muscle. An intermediate condition of sensoria, indicated by an increase in number as compared with wingless, was, in almost every such intermediate studied, associated with non-degenerate wing muscles. The one most notable exception is a case in which there is so little degeneration of muscle that degeneracy may be questioned.

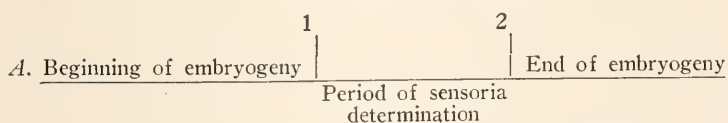
Another correlation is apparent between intermediate sensoria and quantity of muscle, for in general increased sensoria are associated with a relatively large amount of wing muscle. An intermediate of particular interest in this connection is one in Table VI, slide 1061 *c* (1), wing .5, ocellar development .94, and wing muscle development .99 with 14 and 15 sensoria, which is almost normal for the winged. It has been difficult to relate the time-of-determination theory to the number of sensoria. On the basis of this theory, if sensoria were determined very late, as might be suspected from their correlation with wing muscle, one would expect more sensoria than is characteristic of typical wingless individuals in at least some of the intermediates with only .01 wing development which presumably changed to the winged condition very late in their embryogeny. This condition is not met in any of the intermediates with .01 wing development, yet there are many of these.

The data on intermediates, if the order of determination is wings, ocelli, and wing muscle as proposed by Stiles (1938), permit the interpretation that the greater number of sensoria characteristic of the winged aphid is determined neither very late nor early, but somewhere between these extremes. Increased sensoria cannot be determined early in development or they would be found more generally in intermediates with degenerate wing muscle; neither does it seem probable that they are determined very late or some of the aphids with a low degree of intermediacy (little development of wings) and non-degenerate muscles would possess intermediate antennae. With the exception of one, the most muscle found in intermediates with degenerate wing muscle is .57

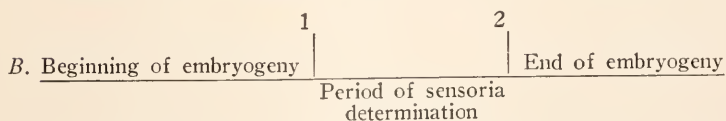
of normal development, indicating a change to wingless before the late stages in the segregation of this organ. The one exception of much muscle associated with degeneracy is P-27 of Table V. Unfortunately the sensoria number and antennal color of this aphid are not known as it was selected for a typical winged aphid and it was only after a histological study that intermediacy was recognized. If the antennae had been the light color of wingless aphids, it would seem as though that feature would have been observed, although it could have been overlooked for they were not examined with a microscope. Of those aphids in this investigation which were rated as to antennal characters and in which there were degenerate wing muscles, it may be postulated that development in the direction of wings was not far enough advanced before a change to wingless occurred for increased sensoria to be determined. All aphids in which the non-degenerate muscles are correlated with a low degree of wing development and a small amount of wing muscle are individuals in which the change to wings came too late in their embryonic development for an increase in sensoria to be determined. Only intermediate-winged aphids which change from a wingless to a winged condition early enough in their embryogeny to have wing muscles comparatively well developed pass through that part of the developmental period in which an increased number of sensoria is determined. This hypothesis may be illustrated by using a horizontal line to represent the entire embryogeny with vertical lines intersecting at various points to set off periods of time, as below.

Embryonic order of determination—wings, ocelli, wing muscle.

Aphid begins embryogeny wingless and changes to winged.



Aphid begins embryogeny winged and changes to wingless.



In *A*, intermediates with a small amount of wing muscle change to the winged condition at a time later than the interval marked off by the perpendicular line number 2, and therefore do not possess more sensoria than the typical wingless aphids. In *B*, intermediates with degenerate muscles as a consequence of a winged-to-wingless transition, change to

wingless before the time interval designated by 1 has been reached, and therefore do not possess an increased number of sensoria. Of the aphids in this study only those which changed from wingless to winged early enough to have considerable wing muscle passed through that part of the developmental period between the intervals 1 and 2.

These studies have led to the inference that the wings, ocelli, and wing muscles have their determination spread over considerable time, whereas the determination of the sensoria takes place in a relatively short time. An extensive series of intergradations in the development of the wings, ocelli, and wing muscles as contrasted with the decidedly limited series of the sensoria suggests this. From this analysis one concludes that increased sensoria are not determined until after the initial determination of wings, ocelli, and wing muscles; the former, however, complete their segregation before the latter.

From the data, it is concluded that the reason why aphids of this study with degenerate wing muscles did not usually possess increased sensoria is because the development in the direction of wings stopped short of the time when a greater number of sensoria would have been determined. Intermediates with considerable non-degenerate wing muscle changed from the wingless to the winged condition early enough to include in their development the period over which increased sensoria were determined. This analysis does not involve the inference that sensoria develop as a response to wing muscle, for in many cases the data show considerable wing muscle development without an intermediate number of sensoria. However, it is assumed that the time when increased sensoria in the embryogeny of the winged aphid are determined is indicated by the quantity of wing muscle. With the knowledge of the time when wing muscle is segregated in embryonic development as a point of reference, the time when sensoria characteristic of the winged aphid are determined can be ascertained provided that differentiation of the sensoria follows determination closely. According to this analysis, sensoria typical for winged forms are determined after wings, ocelli, and wing muscles.

SUMMARY

Dark antennal color and increased sensoria of winged aphids are considered to be characters closely correlated in development, for in general when there was a darkening of antennae there was a corresponding increase in the number of sensoria. In practically all cases increased sensoria were correlated with a relatively large amount of non-degenerate wing muscle. It is concluded that embryonic determination of dark antennal color and increased sensoria takes place in a comparatively

short period of time as compared with that of wings, ocelli, and wing muscle. The data make it seem probable that dark antennal color and extra sensoria, characteristic of the winged aphid, are determined after the wings, ocelli, and wing muscle; the former, however, complete their segregation before the latter.

LITERATURE CITED

- SHULL, A. F., 1933. The time of embryonic segregation in aphids as determined from intermediate types. *Proc. Nat. Acad. Sci.*, **19**: 164.
- SHULL, A. F., 1937. The production of intermediate-winged aphids with special reference to the problem of embryonic determination. *Biol. Bull.*, **72**: 259.
- STILES, KARL A., 1938. Intermediate-winged aphids and the time-of-determination theory. *Biol. Bull.*, **74**: 430.

SOME PHYSIOLOGICAL PROPERTIES OF DEXTRAL AND OF SINISTRAL FORMS IN *BACILLUS* *MYCOIDES* FLÜGGE

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The attention of biologists has long been concentrated on the study of dextral and of sinistral forms in populations of the same species. Recently Ludwig (1932, 1936) in two extensive reviews has summarized the numerous scattered observations in this field. Among studies of dextrality and sinistrality in the broad sense it seems useful to treat separately a distinct group of observations on spiral structures which are spread widely in different representatives of both plant and animal kingdoms. One of the basic attributes of spiral structures is their property of undergoing genotypic inversion with comparative ease, as has been shown, for instance, by Boycott, Diver, Hardy and Turner (1929) in the mollusk *Limnaea peregra*. In almost all cases when experimental work is carried out with organisms possessing a spiral form, it is possible to detect among the individuals of typical structure a few hereditary inverse specimens.

It may be supposed that the direction of the spiral is determined by some substance which is labile in the sense that it is able to undergo inversion of configuration with comparative ease. Such an idea is due to Koltzoff (1934) and to Needham (1934), who wrote that it is possible that the origin of dextrality and sinistrality in the eggs of certain snails, which later appear in the direction of spiral twist of their coil, is due to stereochemic properties of the protein molecules composing them. At present these relations are very obscure, and it seems essential to record accurately which physiological properties of dextral and sinistral spiral forms of organisms are identical and which are not. Such analysis is a necessary step in the attempt to comprehend the mechanism of morphologic inversions.

The Material and its Morphologic Properties

For the investigation of physiological peculiarities of dextral and sinistral spiral forms *Bacillus mycoides*, described by Flüge in 1886, was chosen. Various cultures of dextral and sinistral forms of this bacterium have been received from the Microbiologic Institute of the

Academy of Sciences (Moscow). Under natural conditions a form of *Bacillus mycoides* is usually met with which, when grown on the surface of agar-peptone medium, produces colonies spirally twisting to the left, i.e., counter-clockwise. The inverse form of this microbe, producing dextral coils in growing, rarely occurs. Isolated colonies consisting wholly of dextrally twisted individuals are sometimes found in soils. The dextral form of *Bacillus mycoides* was first recorded by Gersbach (1922), and in experiments with agglutination he established that the dextral and sinistral strains of this organism are perfectly identical in all their properties. Later dextral strains among a number of sinistral ones were observed by Oesterle (1929) and by Lewis (1932). The dextrality and sinistrality in *Bacillus mycoides* is a hereditary feature inasmuch as dextral forms are always obtained from dextral forms and sinistral forms from sinistral after numerous transfers on peptone-agar.

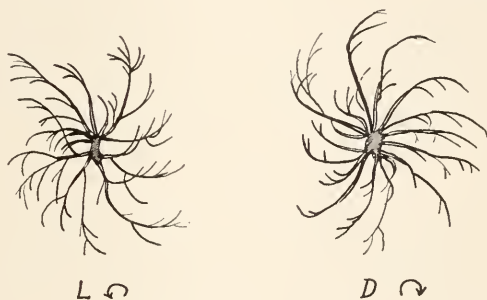


FIG. 1. Dextral (D) and sinistral (L) colonies of *Bacillus mycoides*, 18 hours after inoculation on peptone-agar in Petri dishes, at 29° C. Diameter of the colonies attains 20 mm.

Figure 1 represents the appearance of colonies formed by the dextral and sinistral forms of *Bacillus mycoides* on peptone-agar. With the introduction of a small quantity of inoculating material into the centre of a Petri dish of agar-peptone, the growth begins and the thin filaments of the growing culture of the L-form steadily deviate to the left and those of the D-form to the right.

It can be shown that the spiral form of the colony of *Bacillus mycoides* is a secondary feature which is the result of the primary spiral structure of the growing cells of the filament. If one stains the growing cells of the filaments on the surface of agar by neutral red or by toluidin blue (1:5000), and then examines them under the microscope, one sometimes detects the twisting of two filaments on encountering one another on the surface of the agar. A number of such observations prove that during the growth of the filament motions of two kinds take place simul-

taneously: first, the elongation and, second, the twist around the axis of the filament. As a result of the combination of these two motions, the structure of the individual cell and with it the structure of the whole filament forms a spiral. Similar observations have been made also by Stapp and Zycha (1931) and by Roberts (1938).

If during the free growth of a filament on the agar surface, the filament rotates around its longitudinal axis counter-clockwise, as a result of interaction with the firm surface of the agar the growing filament will also steadily coil in a counter-clockwise direction. Consequently, the secondary sinistral coil of the growing colony of bacteria will arise as a result of the primary sinistral spiral growth of the cells of the filament. This viewpoint is confirmed by the following observations. It appears that a certain consistency of culture medium is necessary for the typical spiral growth of colonies of *Bacillus mycoides* (Pringsheim and Langer, 1924; Hastings and Sagen, 1933). The latter authors state that on agar of usual strength the growth of *Bacillus mycoides* spreads from the place of inoculation in the form of coarse filaments which twist counter-clockwise, forming a symmetrical pattern. On less consistent agar this pattern disappears, and the growth becomes diffuse.

Methods

The strains used in this investigation were cultivated during the last five years under standard laboratory conditions. It is recorded that long cultivation and repeated transfer of the strains of *Bacillus mycoides* level many differences in morphological and physiological characteristics observed immediately after the isolation of the strains from natural conditions (Kononenko, 1935), and only those of them are retained which are due to the hereditary pattern of organization of the strain. This circumstance is of significance in the comparison of physiological properties of dextral and sinistral forms.

Experiments were made under sterile conditions. The bacteria were cultivated in Petri dishes on a solid medium of the following composition: K_2HPO_4 —0.05 per cent; $MgSO_4$ —0.03 per cent; NaCl—0.03 per cent, peptone—1 per cent and agar-agar—2 per cent. In some experiments the effect of optical isomers of amino acids upon the bacteria was studied.¹

¹ In this work preparations of the following origins were used: *d*-valine, *l*-valine, *l*-aspartic acid and *dl*-aspartic acid: Schuchardt Co. (Görlitz); *d*-leucine (reinst), *l*-leucine (reinst), *l*-histidine monochlorhydrate, *d*-histidine monochlorhydrate, *l*- β -phenyl- α -alanine (reinst), *d*- β -phenyl- α -alanine (reinst): Fraenkel and Landau (Berlin); *d*-arginine monochlorhydrate (recrystallized from 80° alcohol), *dl*-arginine monochlorhydrate (isolated by Dr. M. Lissitzin): Protein Laboratory of the Academy of Sciences (Moscow).

Relations of Growth in Dextral and in Sinistral Strains to Temperature

The first group of experiments was devoted to the study of the effect of temperature on the growth of dextral and sinistral strains of *Bacillus mycoides*. Before the inoculation *Bacillus mycoides* was always cultivated on solid agar in Petri dishes at 20° C., while for the last forty-eight hours the culture was maintained at 15° C. From such cultures, accustomed to moderate temperatures, an inoculation into solid or liquid medium was made and the growth was followed usually at the following temperatures: 20°, 24°, 28° and 32° C. In cases when it is not specially mentioned, the experiments were made with the sinistral strain, L-1, and with the dextral strain, D-1. The basic conclusions arrived at in the work with these strains were further confirmed upon other strains, as will be described below.

Figure 2 gives the results of three series of experiments made in Petri dishes. Into the centre of each dish was introduced a small quantity of inoculating material which formed a circle with a diameter of 0.5 mm. Twenty hours after the beginning of the growth the diameter of the colony was measured and as a rule it had attained about 6 mm. at 20° C., and about 25 mm. at 32° C.

Observations have shown that at this time the growth proceeds at all temperatures according to a geometric progression. In Fig. 2 the size of colonies of the dextral and sinistral strains growing at 20° C. was taken as a unit, and the size at other temperatures is expressed in relative values. The fundamental conclusion which may be drawn from Fig. 2 is that the sinistral and dextral strains of *Bacillus mycoides* differ sharply from one another in the relation of growth to temperature. If the rate of growth of the usual sinistral strain increases exponentially with the rise of temperature, as is typical for the majority of biological processes, in the inverse dextral strain the phenomenon of "heat injury" may be observed. With the rise of temperature the velocity of growth of the dextral strain increases always more and more slowly.

This observation has necessitated a more thorough study of the phenomenon of "heat injury" in the dextral strain in additional material. In the preceding experiments the growth of the so-called R-forms of bacteria, producing sinistral (LR) or dextral coils (DR) was studied. As a result of "dissociation" both dextral and sinistral strains of *Bacillus mycoides* develop into smooth ones, the so-called S-forms, deprived of coiling and forming during their growth on solid medium flat spheric colonies (LS and DS) (for literature see Arkwright, 1930). In their external appearance S-forms of dextral and sinistral strains are certainly indistinguishable from one another. Investigations devoted to

the study of dissociation in *Bacillus mycoides* have shown that the transition of the R-form into S-form is very easily accomplished, while the reverse transformation of the S-form into the R-form is extremely

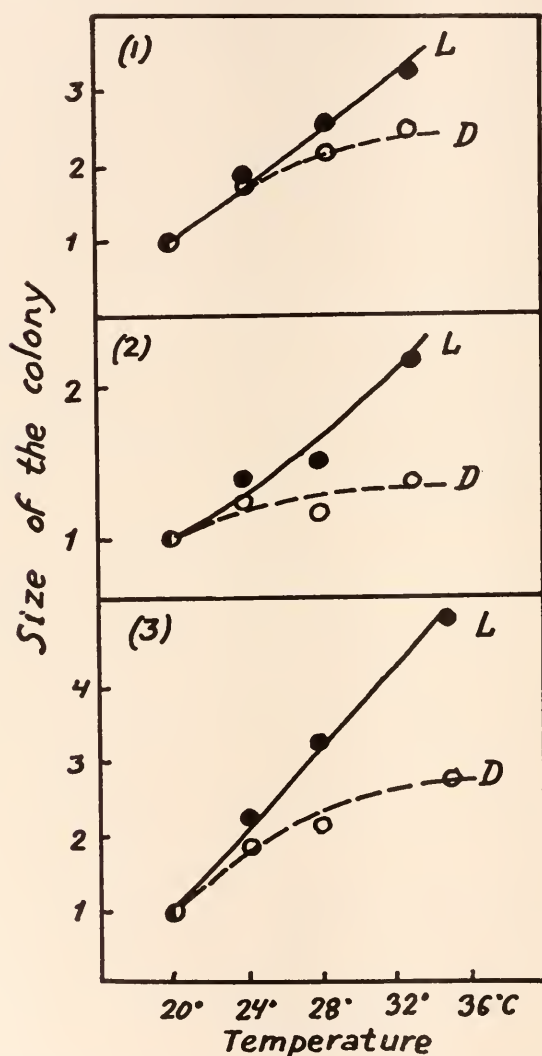


FIG. 2. Temperature relation of growth in the dextral (DR) and sinistral (LR) strains of *B. mycoides* on the solid medium. Each point represents an average for three dishes.

unusual (Stapp and Zycha, 1931; Lewis, 1932; Dooren de Jong, 1933).

An attempt was made to find out whether the specific differences in the dependence of growth on temperature in the dextral and sinistral

strains which were observed on the R-forms will also be maintained in the case of the S-forms. Experiments on the relation of growth to temperature in LS and DS forms of *Bacillus mycoides* growing on solid

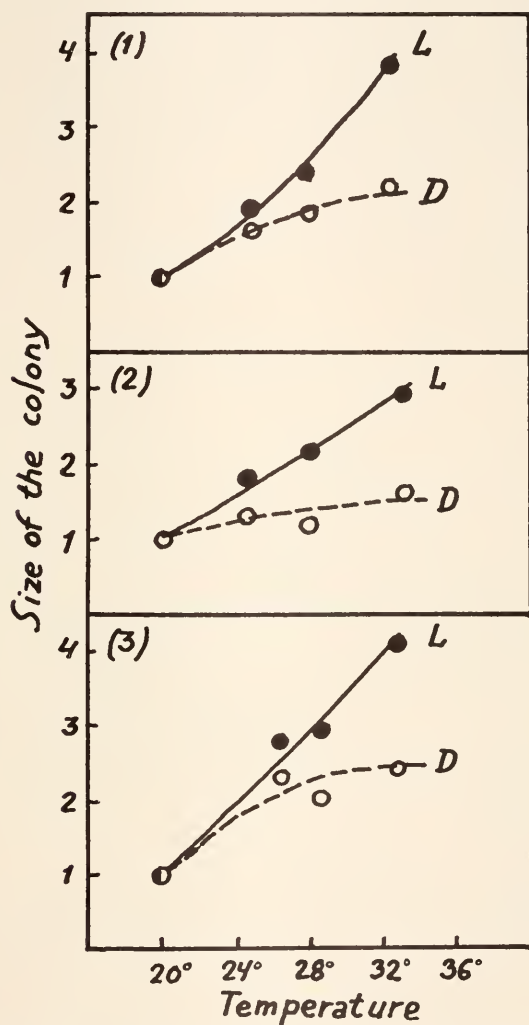
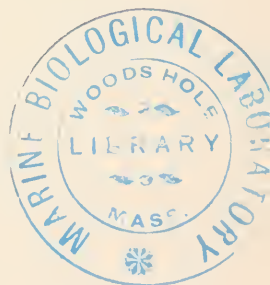


FIG. 3. Temperature relation of growth in the smooth dextral (DS) and sinistral (LS) strains of *B. mycoides* on the solid medium. Each point represents an average for three dishes.

medium were made according to the previous plan and the results of the three series of observations are given in Fig. 3. It is evident that the phenomenon of heat injury in growth which was found to be char-



acteristic for the dextral strains is practically equally marked both in the R-form and in the S-form of the bacteria.

Further experiments on the relation of growth to temperature in DS and LS strains were made with liquid medium of the following composition: K_2HPO_4 —0.05 per cent, $MgSO_4$ —0.03 per cent, $NaCl$ —0.03 per cent, glucose—1.0 per cent, d-arginine—0.05 per cent. The medium in this and in further experiments was sterilized without arginine, and a powder of arginine was aseptically added to it later. The bacteria were cultivated in test-tubes previously sterilized by dry heat and containing 1 cc. of aseptically added sterile culture medium. The inoculating material was prepared in the following manner. Two loops of

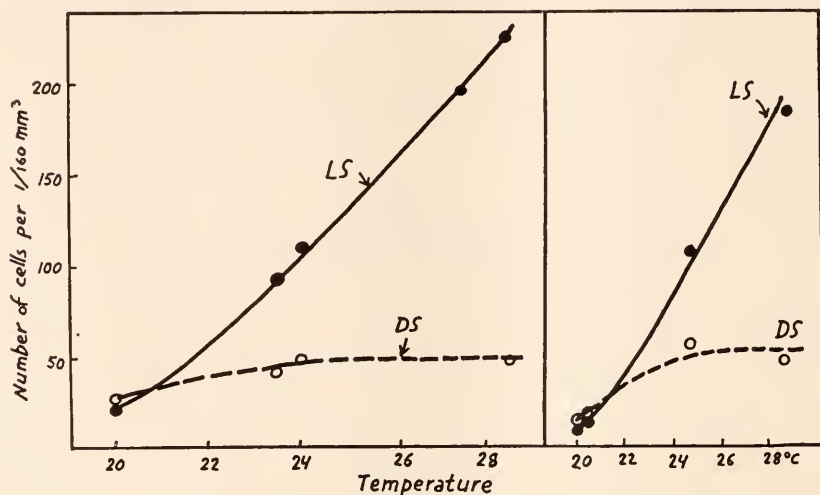


FIG. 4. Temperature relation of growth in the DS and LS strains of *Bacillus mycoides* in the liquid medium. Each point represents an average for four observations.

bacteria growing in Petri dishes on agar-peptone at 20° C. were shaken up in 3 cc. of sterilized bidistilled water, and two drops of this mixture were aseptically introduced into each of the experimental test-tubes, which were subsequently kept at different temperatures (20°, 24°, 26° and 28° C.). The measurement of growth consisted in counting the number of cells in a Thoma chamber under the microscope forty hours after the inoculation. In Fig. 4 the relation of growth to temperature is given as observed in two series of experiments upon the LS and DS forms of *Bacillus mycoides* in the liquid medium. It may be pointed out that in the liquid medium the velocity of growth of the sinistral strain rises exponentially with the increase of temperature, while in the dextral strain the characteristic heat injury is distinct in the range 24°–28° C

In the light of all these experiments it is certain that the dextral (D-1) and sinistral (L-1) strains of *Bacillus mycoides* under examination possess distinctly different relations of growth to temperature and that these distinctions are retained both in solid and in liquid medium, both in R and S modifications. However, are these observations sufficient for the belief that the differences observed are actually connected with the dextrality and sinistrality of the strains studied? In order to

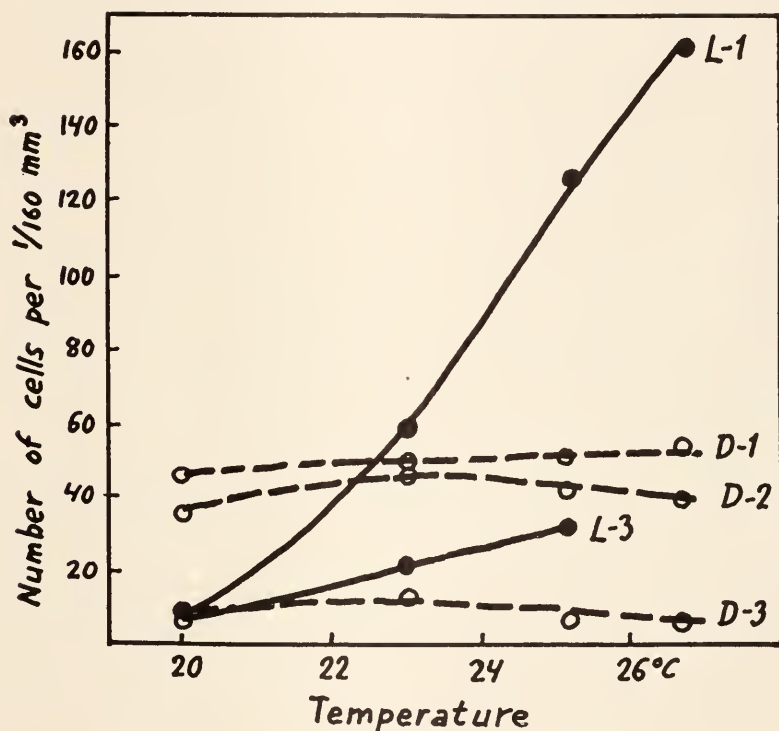


FIG. 5. Temperature relation of growth in the smooth sinistral (L-1, L-3) and dextral (D-1, D-2, D-3) strains of *Bacillus mycoides* in the liquid medium. Each point represents an average for three observations.

answer this question it is necessary to observe a great number of various strains of *Bacillus mycoides*. To this end one additional sinistral strain, L-3, and two new dextral inverse strains, D-2 and D-3, were chosen. All these strains had been cultivated under laboratory conditions for five years. S-modifications of various strains were used for experimentation for the reason that it is easier to prepare the suspension for inoculation from them. The results of experiments carried out in liquid medium with five strains of *Bacillus mycoides* of different origin are given in Fig. 5. It may be pointed out that the differences in the

relation of growth to temperature are more distinctly expressed in the two strains which were previously compared, i.e., in L-1 and D-1. However, if one examines the temperature range 23°–26° C., it may be observed that there is a specific distinction between all the dextral and all the sinistral strains. In dextral strains (D-1, D-2, D-3) in this temperature range the rate of growth does not rise with the rise in temperature, while in both sinistral strains (L-1 and L-3) the rate of growth rises considerably with the increase in temperature.

Physiological Difference between the Sinistral Strains and the Dextral Strains of Bacillus mycoides (Lewis Reaction)

Lewis (1933), working in Texas with dextral and sinistral strains of *Bacillus mycoides*, recorded a specific physiological distinction between them. It is known that *Bacillus mycoides* possesses the capacity of decomposing glucose and saccharose with the formation of acids. The production of acids on glucose is similar in sinistral and in dextral strains. This similarity does not hold true for the decomposition of saccharose. Lewis writes that the rapid formation of acid on saccharose is, on the whole, connected with the sinistral direction of spiral growth, while the arrest of this reaction is correlated with the dextral twist of the filaments.

It was decided to repeat these experiments of Lewis with the strains of *Bacillus mycoides* under investigation. S-forms of different strains were used in these experiments. Two per cent of saccharose was added to agar-peptone of usual composition and also some weak (0.02 per cent) solution of phenol red (according to Clark). The latter had an orange tint at pH 7.7, which is optimal for the growth of *Bacillus mycoides*. The microbes were cultivated at 28° C. Nineteen hours after the beginning of the experiment the color of the sinistral strains differed very sharply from that of the dextral strains. The sinistral strains, L-1 and L-3, showed rapid production of acid on sucrose. The reaction of the colony was distinctly acid to phenol red (pH about 6.8). The dextral strains, D-1, D-2 and D-3, showed no production of acid on sucrose. The reaction of the colony was alkaline to phenol red (pH about 8.4).

The results of Lewis are therefore confirmed and it may be pointed out that we used S-modifications while he used R-modifications of the bacteria. This confirmation includes also the following detail. The dextral strains are, without exception, unable to produce acid on sucrose, while almost all sinistral strains readily form acid on sucrose. To this general rule, Lewis noted one exception and I have also come across one doubtful case.

The reaction of Lewis may be considered to be a certain fermentative weakness of inverse dextral strains as compared to typical sinistral ones.

On the Relation of Morphological Inversion to Molecular Inversion

The phenomenon of heat injury of inverse dextral strains of *Bacillus mycoides* coincides in a remarkable way with the heat injury taking place on cultivating different lower organisms on unnatural isomers of amino acids described by Gause and Smaragdova (1938). When the yeast cells *Torula utilis* are grown on the usual isomer of leucine, which enters into the composition of all living organisms, the velocity of growth rises exponentially with an increase of temperature, but when they are cultivated on the unnatural isomer of leucine the velocity of growth increases always more and more slowly with the rise of temperature.

It was decided to repeat this observation of Gause and Smaragdova (1938) on different amino acids and on different organisms. The experiments were made on the growth of *Torula utilis* on optical isomers of leucine, histidine, phenyl-alanine and valine. Cultivation took place in sterile test-tubes containing 1 cc. of liquid medium of the following composition: KH_2PO_4 —0.06 per cent, MgSO_4 —0.03 per cent, glucose—1.0 per cent, amino acid—0.4 per cent (the latter being aseptically introduced into the sterile medium). The inoculating material was taken from the solid medium, and from it was made a mixture of standard density (50 cells in 1/160 cu. mm.), a few drops of which was then added to liquid in the test-tubes. The counting of the number of cells in the Thoma chamber was made 40 hours after the beginning of the experiment. According to Gause and Smaragdova (1938) this time lies within the limits of the logarithmic phase of growth of *Torula*.

The results of our experiments are given in Fig. 6. Typical heat injury in the temperature range of 18°–20° may be observed in the growth of *Torula utilis* on unnatural isomers of leucine and of histidine. In the case of growth of unnatural isomers of valine and phenyl-alanine such injury is not observed.

Further experiments were made with the growth of *Aspergillus niger* on optical isomers of leucine and valine. The composition of culture medium was as follows: KH_2PO_4 —0.35 per cent, MgSO_4 —0.17 per cent, glucose—1 per cent, amino acid—0.4 per cent. Cultivation was conducted under sterile aerobic conditions in 2 cc. of the liquid. Seventy hours after the beginning of the experiment the wet weight of the mycelium of *Aspergillus*, which was previously drained on filter paper, was measured. The results of experiments given in Fig. 7 coincide completely with the observations made on *Torula utilis*.

Summarizing the results heretofore described, it is evident that during the growth of different lower organisms on optical isomers of certain amino acids the effect of temperature on the growth on natural and on

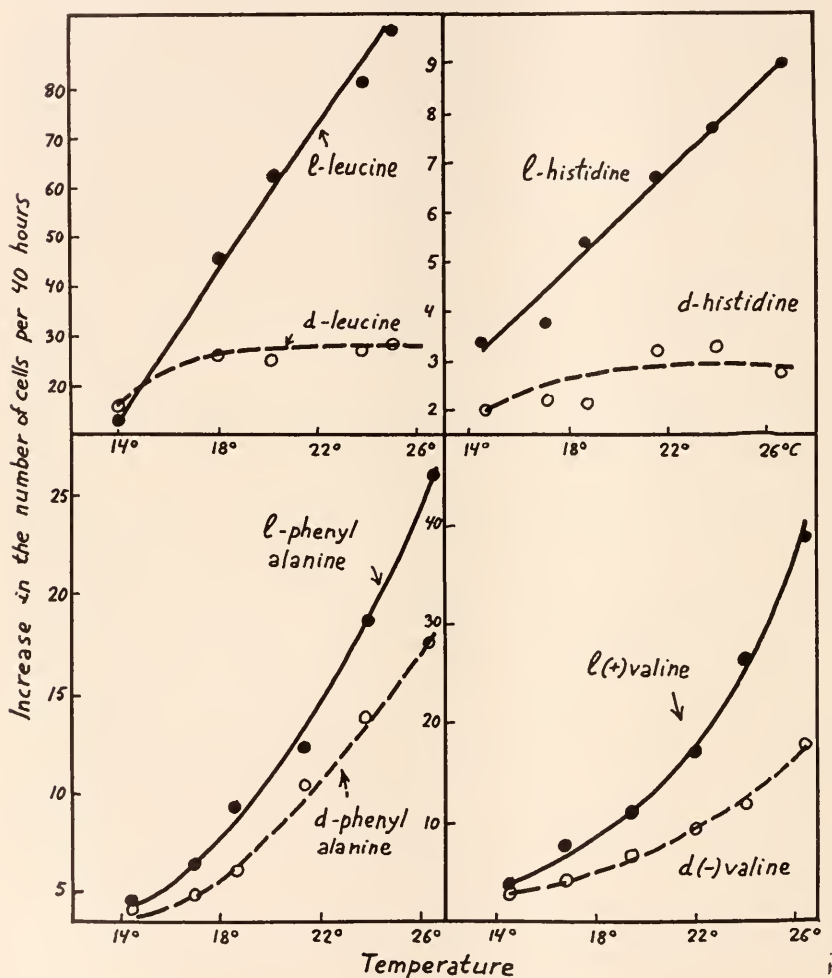


FIG. 6. Growth of the yeast *Torula utilis* on optical isomers of various amino-acids at different temperatures. On the ordinates is plotted the increase in the number of cells per 40 hours in relation to control (the initial density at inoculation, which is taken for a unit). Each point represents an average for two similar cultures.

unnatural isomers proves to be different. The velocity of growth on natural isomers rises exponentially with an increase of temperature. On the other hand, in the same temperature range, the velocity of growth

on the unnatural isomer of some amino acids increases with the rise of temperature always more and more slowly. A similar temperature-velocity curve was observed in the inverse dextral strains of *Bacillus mycoides* while they were being grown on the natural substrates. It follows from this type of curve that there is an inhibitive factor in growth and that the intensity of the inhibition rises with the rise of temperature.

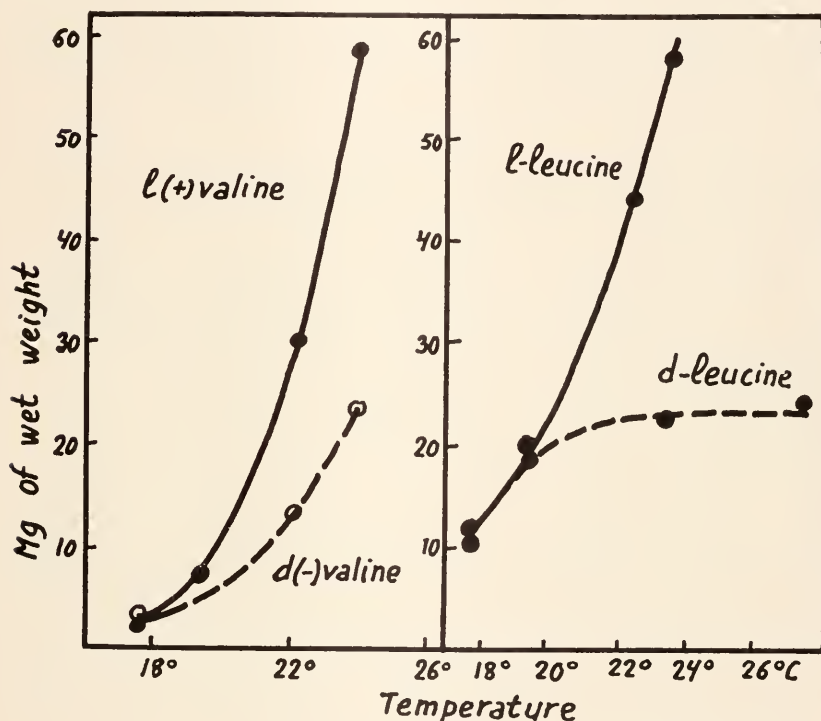


FIG. 7. Growth of *Aspergillus niger* on optical isomers of leucine and valine at different temperatures. Each point represents an average for two similar cultures.

It may be conjectured that in the first series of investigations the unnatural isomer of amino acid dissolved in the culture medium surrounding the cell causes a depression of growth because its steric configuration does not coincide with the steric configuration of the basic constituents of protoplasm. In the second series of investigations one may suppose that the depression of growth in the morphologically inverse form takes place because inside the cell there is an inverse of the normal optical isomer of some organic substance participating in the

determination of the form of cells. The depression of growth could thus take place because the steric configuration of the organic substance in question does not coincide with the steric configuration of other constituents of protoplasm.²

Growth of Dextral and Sinistral Strains of Bacillus mycoides on Optical Isomers of Certain Amino Acids

All further experiments were carried out with the strains L-1 and D-1. It is to be pointed out that not all amino acids by far are adequate for the nutrition of *Bacillus mycoides*. Thus, for instance, on aspartic

TABLE I

The growth of sinistral (LR) and of dextral (DR) forms of *Bacillus mycoides* on optically isomeric arginines.

Amino acid	Number of cells in LR	Growth on <i>d</i> -arginine if growth on <i>dl</i> -arginine is taken as a unit	Number of cells in DR	Growth on <i>d</i> -arginine if growth on <i>dl</i> -arginine is taken as a unit
<i>d</i> -arginine.....	31.3	} 1.99	19.7	} 1.59
<i>dl</i> -arginine.....	15.7		12.4	
<i>d</i> -arginine.....	28.9	} 1.98	21.2	} 1.61
<i>dl</i> -arginine.....	14.6		13.2	

acid only a small increase of the number of cells at the beginning of cultivation can be observed, and the further growth soon stops. The experiments have shown that on l-aspartic acid both the smooth sinistral (LS) and the smooth dextral (DS) strains of *Bacillus mycoides* grow better than on the racemic dl-aspartic acid. Among the amino acids used by us the only substance giving unlimited growth of *Bacillus mycoides* was arginine.

The experiments were made at 24° in test tubes with liquid medium of the following composition: K₂HPO₄—0.05 per cent, MgSO₄—0.03 per cent, NaCl—0.03 per cent, glucose—1 per cent and arginine—0.05 per cent. Each number given in Tables I and II is a mean value for five parallel observations. In all cases the number of cells in 1/160 cu. mm. was counted 48 hours after the beginning of the experiment.

Both the dextral and sinistral forms of *Bacillus mycoides*, in both twisted and smooth modifications, grow better on natural d-arginine

² This hypothesis is further supported by recent observations of Bruckner and Ivanovics (*Zeitschr. f. physiol. Chem.*, **247**: 281, 1937). They observed that in the composition of the capsule of some bacteria an unnatural isomer of glutamic acid participates.

than on the racemic *dl*-arginine. Since racemic arginine in weak watery solution consists of the dextrorotatory and of the laevorotatory isomers, the lessened growth of *Bacillus mycoides* on this preparation may be explained by the fact that the unnatural laevorotatory isomer, entering into the composition of racemic arginine, is less appropriate for growth than the usual dextrorotatory isomer of arginine. In this relation the properties of dextrally and of sinistrally twisted strains coincide with one another.

Oxidation of Glucose by Dextral and Sinistral Strains of Bacillus mycoides at Different Temperatures

Experiments with the oxidation of glucose were made with the Warburg technique at three temperatures: 22°, 25° and 28° C. Bacteria growing on the solid medium at 20° were removed with a platinum loop

TABLE II

The growth of smooth sinistral (LS) and of smooth dextral (DS) forms of *Bacillus mycoides* on optically isomeric arginines.

Amino acid	Number of cells in LS	Growth on <i>d</i> -arginine if growth on <i>dl</i> -arginine is taken as a unit	Number of cells in DS	Growth on <i>d</i> -arginine if growth on <i>dl</i> -arginine is taken as a unit
<i>d</i> -arginine	40.1	} 1.26	66.8	} 2.15
<i>dl</i> -arginine	31.9		31.0	
<i>d</i> -arginine	43.5	} 1.87	45.5	} 1.38
<i>dl</i> -arginine	23.3		33.0	

and a suspension of them was made on a salt solution of the following composition: K_2HPO_4 —0.05 per cent, $MgSO_4$ —0.03 per cent, NaCl—0.03 per cent. Experiments were made with 0.5 cc. of liquid and the oxidation of 0.5 per cent glucose was studied in the atmosphere of oxygen. Into the side tube of the Warburg vessel was poured a 5 per cent solution of KOH for the absorption of carbonic acid. The experiment at every temperature was always begun with a fresh suspension of the standard concentration. Five Warburg manometers were used simultaneously. One of them served as thermobarometer, two of them contained the suspension of sinistral (LS) bacteria, and two others the suspension of dextral (DS) bacteria. Fifteen minutes were allowed for temperature equilibrium, and then the readings of oxygen consumption were made at 20-minute intervals for 1½ hours. On the basis of these data the mean values of oxygen consumption at different temperatures were calculated.

Figure 8 gives the results of two series of experiments. As is characteristic of biological processes generally, the velocity of respiration rises exponentially with the rise of temperature, and practically in equal degree both in the sinistral and in the dextral strains of *Bacillus mycoides*.

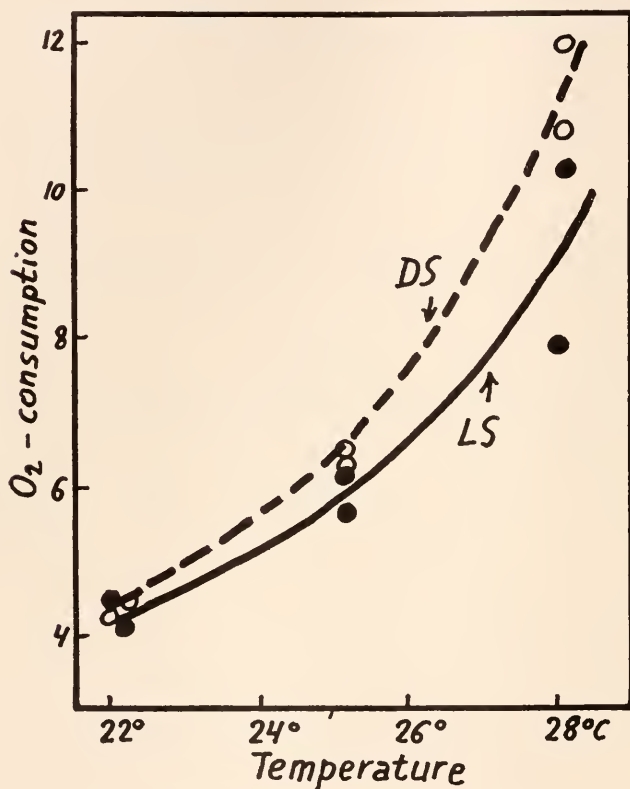


FIG. 8. Oxygen consumption by suspensions of dextral (DS) and sinistral (LS) strains of *Bacillus mycoides* at different temperatures. Oxygen consumption per 20 minutes is expressed in divisions of the standard manometer and represents an average for four consecutive readings.

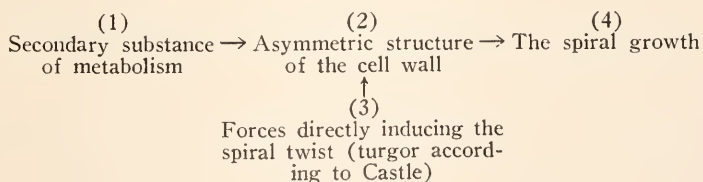
Consequently the phenomenon of heat injury in the dextral strain, which is characteristic for its growth, is absent in respiration on glucose.

DISCUSSION

Investigations of different physiological properties of dextral and of sinistral strains of *Bacillus mycoides* show that in the basic features of their metabolism these strains are alike. Both in the sinistral and in the dextral strains natural dextrorotatory arginine proves to be more

suitable material for growth than the racemic arginine. Both the sinistral and the dextral strains oxidize natural dextrorotatory glucose equally well and with the same temperature coefficients. The specific difference between dextral strains and sinistral ones is consequently connected not with the inversion of the fundamental protoplasmic system but with differences in some secondary substances determining the cellular form. Castle (1936) has recently undertaken the study of the spiral growth of the cell in *Phycomyces*. He advocates the idea that the spiral structure of the growing cell wall is not strictly predetermined beforehand, but depends on interaction of forces acting in the region of growth. The twist of the growing elastic elements of the wall may be the result of their resistance to the pressure of turgor, as has been shown by Castle (1936) with a special model. It is interesting that if the elastic elements of the wall are distributed symmetrically, in 50 per cent of the experiments with the model dextral spirals are obtained, and in 50 per cent sinistral spirals. As the sinistral direction of spirals is typical for *Phycomyces*, it is evident that the reason for this fact, if one adopts the explanation of Castle, is to be found in some sinistral distribution of elastic elements of the cell wall, which later, under the action of turgor, leads to the formation of sinistral spirals.

As Castle himself points out, the mechanism of spiral growth in different organisms can be different, and one cannot directly transfer his considerations to the bacteria, the more so since the cell wall of the latter consists of some specific protein material which is formed in close connection with the cellular protoplasm (John-Brooks, 1930). It is only important to realize that in the spiral growth of the cell wall there is, first, a system of forces directly inducing the spiral twist (like the turgor in the model of Castle) and, second, that there is some pre-existing asymmetric system (distribution of the elastic elements of the wall), the dextrality or sinistrality of which brings on the dextrality or sinistrality of the spiral growth. Whatever the actual mechanism of spiral growth may be, one can conceive the following general scheme of the formation of spiral structure of the cell wall:



One may conjecture that the inversion of the direction of spirals in *Bacillus mycoides* is related to optical inversion of some secondary substance in metabolism. The latter brings on the inversion of some struc-

tures in the cell wall, and during the interaction of these with the forces inducing the spiral twist the inversion in the direction of the spiral growth of the cells is brought about.

In conclusion it is to be pointed out that the optical inversion of the secondary substances determining cellular form is judged from indirect evidence, and it is consequently highly desirable to check this conclusion by direct chemical methods.

SUMMARY

1. The investigation of different physiological properties of dextral and of sinistral forms of *Bacillus mycoides* shows that in the basic features of their metabolism these strains coincide with one another. Both in sinistral and in dextral strains the natural dextrorotatory arginine proves to be more suitable material for growth than the racemic arginine. Both the sinistral and the dextral strains oxidize the natural dextrorotatory glucose equally well and with the same temperature coefficient.

2. The specific difference between the dextral and the sinistral strains is not consequently connected with the inversion of the basic protoplasmic system, but with differences in some secondary substances determining the cellular form. On the basis of the study of the phenomenon of heat injury in the growth of morphologically inverse dextrally twisted strains of *Bacillus mycoides* the hypothesis is advanced that some organic substance, participating in the determination of the cellular form, has undergone an optical inversion of configuration in the dextral strains.

REFERENCES

- ARKWRIGHT, I. A., 1930. Variation. A System of Bacteriology. Vol. 1, p. 311.
BOYCOTT, A., C. DIVER, S. HARDY AND F. M. TURNER, 1929. The inheritance of sinistrality in *Limnaea peregra*. *Proc. Roy. Soc., Ser. B.*, **104**: 152.
CASTLE, E. S., 1936. A model imitating the origin of spiral wall structure in certain plant cells. *Proc. Nat. Acad. Sci.*, **22**: 336.
CASTLE, E. S., 1936. The origin of spiral growth in *Phycomyces*. *Jour. Cell. Compar. Physiol.*, **8**: 493.
DOOREN DE JONG, L. E., 1933. Ueber *Bacillus mycoides* und den Pleomorphismus. *Arch. Mikrobiol.*, **4**: 36.
GAUSE, G. F., AND N. P. SMARAGDOVA, 1938. An analysis of growth of the yeast *Torula utilis* on optically isomeric leucines and valines. *Biol. Zhurn.*, **7**: 399. (In Russian with English summary).
GERSBACH, A., 1922. Ueber die Wendigkeit der Koloniausläufer des *Bacillus mycoides* ("Isomerie" bei Bakterien). *Zentralbl. Bakt., Abt. I*, **88**: 97.
HASTINGS, E. G., AND H. SAGEN, 1933. The effect of physical environment on the cultural character of *Bacillus mycoides*. *Jour. Bact.*, **25**: 39.
JOHN-BROOKS, R. S., 1930. Morphology. A System of Bacteriology. Vol. 1, p. 104.
KOLTZOFF, N. K., 1934. Genetics and physiology of development. *Biol. Zhurn.*, **3**: 420. (In Russian.)

- KONONENKO, E., 1935. On the basic forms of *Bacillus mycoides* Flügge and their mutual relations. *Microbiol.*, **4**: 4. (In Russian.)
- LEWIS, I. M., 1932. Dissociation and life cycle of *Bacillus mycoides*. *Jour. Bact.*, **24**: 381.
- LEWIS, I. M., 1933. Secondary colonies of bacteria with special reference to *Bacillus mycoides*. *Jour. Bact.*, **25**: 359.
- LUDWIG, W., 1932. Das Rechts-Links-Problem im Tierreich und beim Menschen. Berlin. Springer.
- LUDWIG, W., 1936. Bestimmung und Vererbung der Asymmetrie-form. (Rechts-Links-Problem.) *Verh. Zool. Ges.*, **38**: 21.
- NEEDHAM, J., 1934. Morphology and biochemistry. *Nature*, **134**: 275.
- OESTERLE, P., AND C. A. STAHL, 1929. Untersuchungen über den Formenwechsel und die Entwicklungsformen bei *Bacillus mycoides*. *Zentralbl. Bakt.*, Abt. II, **79**: 1.
- PRINGSHEIM, E. G., UND J. LANGER, 1924. Zur Entwicklungsphysiologie der Kolonien des *Bacillus mycoides* Flügge. *Zentralbl. Bakt.*, Abt. II, **61**: 225.
- ROBERTS, J. L., 1938. Evidence of a rotational growth factor in *Bacillus mycoides*. *Science*, **87**: 260.
- STAPP, C., UND H. ZYCHA, 1931. Morphologische Untersuchungen an *Bacillus mycoides*. *Arch. Mikrobiol.*, **2**: 493.

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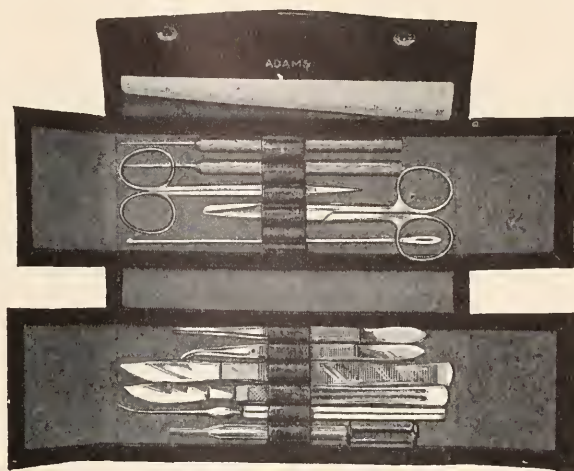
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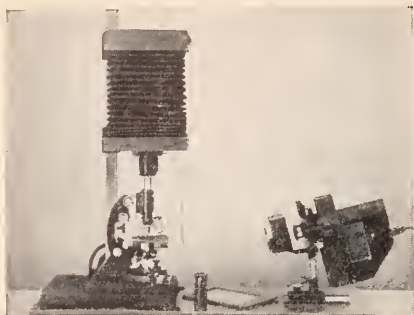
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